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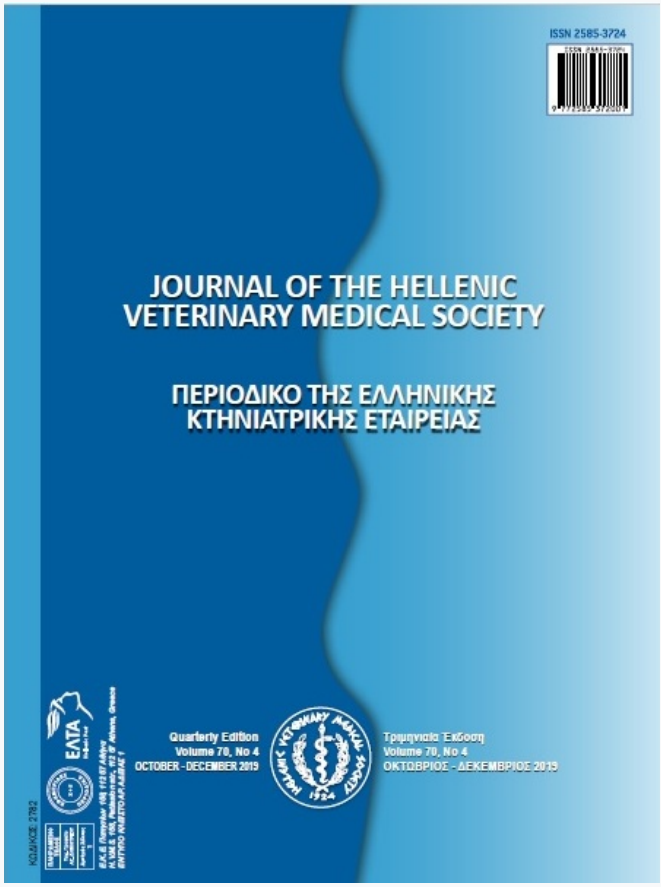


**Whole issue**

*Ελληνική Κτηνιατρική Εταιρεία*

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# ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

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OCTOBER - DECEMBER 2019

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Volume 70, No 4
ΟΚΤΩΒΡΙΟΣ - ΔΕΚΕΜΒΡΙΟΣ 2019

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Τοχ. Γραφείο ΑΓ. ΔΗΜΗΤΡΙΟΥ
Αριθμός Άδειας 1
ΚΩΔΙΚΟΣ: 2782

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H.V.M.S. 158, Patission str., 112 57 Athens, Greece
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E-mail: secretary@hvms.gr

**Εκδότης**
**Ελληνική Κτηνιατρική Εταιρεία**
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A. Tyrpenou
Patission 158, 11257 Athens, Greece
Tel.: +.30.210.8642284
Fax: +.30.210.8645744

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| | | | | Number of specialist veterinarians active in Greece<br>Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα |
|---|---|---|---|---|
| 1 | | ECAR | European College of Animal Reproduction | 2 |
| 2 | | ECAWBM | European College of Animal Welfare and Behavioural Medicine | 2 |
| 3 | | ECAAH | European College of Aquatic Animal Health | 3 |
| 4 | | ECBHM | European College of Bovine Health Management | 3 |
| 5 | | ECEIM | European College of Equine Internal Medicine | 0 |
| 6 | | ECLAM | European College of Laboratory Animal Medicine | 0 |
| 7 | | ECPHM | European College of Porcine Health Management | 3 |
| 8 | | EPVS | European College of Poultry Veterinary Science | 4 |
| 9 | | ECSRHM | European College of Small Ruminant Health Management | 12 |
| 10 | | ECVAA | European College of Veterinary Anaesthesia and Analgesia | 1 |
| 11 | | ECVCN | European College of Veterinary Comparative Nutrition | 0 |
| 12 | | ECVCP | European College of Veterinary Clinical Pathology | 1 |
| 13 | | ECVD | European College of Veterinary Dermatology | 3 |
| 14 | | ECVDI | European College of Veterinary Diagnostic Imaging | 1 |
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| 18 | | ECVO | European College of Veterinary Ophthalmology | 0 |
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| 22 | | ECZM | European College of Zoological Medicine | 1 |
| 23 | | ECVS | European College of Veterinary Surgery | 1 |
| 24 | | EVDC | European Veterinary Dentistry College | 0 |
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# History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veteri- nary Microbiological Institute of Votanicos, from where all mem- bers of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Insti- tute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabil- ities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular mem- bers. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the exe- cution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veter- inary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Compan- ion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (found- ing member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Profes- sors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

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Review article
Ανασκόπηση

# Feline Epilepsy: An update

**R.D. Baka[1], Z.S. Polizopoulou[1]**

[1]*Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

**ABSTRACT:** Epileptic seizures are the most common neurological disorder in the clinical setting. Their etiology is multifactorial and is mainly divided into structural, reactive and idiopathic epilepsy. Structural epilepsy can be caused by vascular events, inflammatory conditions (encephalitis), traumatic injuries, neoplasia, congenital and inherited (degenerative) disorders. Reactive epilepsy is caused by exposure in toxins or metabolic derangements. Although idiopathic epilepsy was thought to be rare in cats, it is now established as a common cause. Epileptic seizures in cats appear with various clinical presentations including generalized, focal with or without secondary generalization epileptic seizures. Diagnostic investigation is crucial in order to establish final diagnosis and to determine the therapeutic plan. Diagnostics include physical and neurological examination with detailed history (drug or toxin exposure), routine hematology (CBC, biochemistry, urinalysis), specific laboratory tests if concurrent or metabolic disease are suspected, advanced diagnostic imaging (CT/MRI) whether intracranial disease is suspected and cerebrospinal fluid (CSF) analysis. Most commonly used antiepileptic drugs (AED) in cats are phenobarbital and levetiracetam. Bromide is contraindicated in cats due to severe respiratory disease caused as an adverse life-threatening reaction. Diazepam is an emergency AED used to eliminate cluster seizures or status epilepticus but it should be avoided as a long-term medication because it has been associated with fatal hepatotoxicity. Gabapentin in another potential antiepileptic drug however its long-term efficacy has to be evaluated. Prognosis depends on the underlying etiology and treatment response. In most cats quality of life is improved and (>50% reduction of epileptic seizures) regardless of etiology. The complete remission of epileptic seizures in cats is rare and most cats should be maintained on anti-epileptic therapy.

***Keywords:*** antiepileptic treatment, epilepsy, feline, seizures

*Corresponding Author:*
Rania D. Baka, Stavrou Voutyra str. 11, 54627, Thessaloniki, Greece
E-mail address: raniadbaka@gmail.com

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## ETIOLOGY OF SEIZURES IN CATS

Epileptic seizures are the most common neurological condition encountered in companion animal practice with an estimated prevalence (in a referral hospital population) of 0.5% to 3.5% in cats (Pakozdy et al., 2010).

Structural epilepsy appears when an underlying structural lesion or disease is identified (Bailey and Dewey, 2009). The major categories of structural epilepsy in cats include vascular events, inflammatory conditions/ encephalitis, infectious etiologies, traumatic injuries, neoplasia, congenital malformations and degenerative conditions (Bailey and Dewey, 2009).

Reactive seizures may result from a wide variety of extracranial causes, including toxins, drugs and metabolic disease (O' Brien, 1998). Similar findings are reported by Rusbridge (2005) and Barnes (2004), where hepatic encephalopathy was the predominant diagnosis in the metabolic group of diseases. However, Schriefl (2008) reports that the percentage of reactive seizures is higher. Kline (1998) indicated that reactive seizures due to hepatic encephalopathy occurred infrequently in cats. Thus, in cats presented with epileptic seizures history should focus on previous drug administration and potential exposure to toxins (O' Brien, 1998).

Hypertension occurs more frequently as a sequel of chronic kidney disease, hyperthyroidism or hypertrophic cardiomyopathy (Kline, 1998). Hypertensive cats more commonly present with retinopathy and blindness, but vascular changes in the brain may lead to arteriosclerosis, focal hemorrhage, epileptic seizures, ataxia, nystagmus, sudden collapse and paraparesis (O' Brien, 1998). Polycythemia can cause epileptic seizures, blindness, abnormal behavior aggression, ataxia, pupillary dilatation and ptyalism (Kline, 1998). Most cats with clinical signs will have a hematocrit of 63% or greater (Khanna and Bienzle, 1994; Watson et al., 1994). Uremia is a relatively uncommon cause of reactive seizures and is usually quite severe before inducing either focal or generalized seizures (Kline, 1998).

While reactive seizures present a serious clinical manifestation for many of these conditions, they can be controlled or eliminated if the underlying cause is detected and treated (O' Brien, 1998).

Inflammatory diseases have been reported to account for 32-44% of histologically confirmed central nervous system diseases in cats (Bradshaw et al., 2004; Rand et al., 1994). The most common cause of meningoencephalitis in cats is feline coronavirus, the etiological agent of feline infectious peritonitis (De Risio et al., 2012). Other known etiologies of feline meningoencephalitis are uncommon and include viruses such as feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline parvovirus, pseudorabies virus/ porcine herpesvirus 1, rabies virus, Borna disease virus, West Nile virus, encephalomyocarditis virus, and protozoal, bacterial, rickettsial, fungal and parasitic agents (Gunn Moore, 2005; Schwab et al., 2007). In a large number of feline cases with central nervous disease, histopathological changes consistent with lymphohistiocytic (non-suppurative) meningoencephalitis are found (Schwab et al., 2007). Although, lymphohistiocytic meningoencephalitis is usually suggestive of viral infection, the causative agent is often not identified (De Risio et al., 2012). In lymphohistiocytic meningoencephalitis of unknown origin the clinical signs appear at a young age (2 years or less), and the progression is no longer than a couple of weeks (Gunn Moore, 2005; Rand et al., 1994). De Risio (2012) presented different evidence regarding the disease. More specifically, cats with lymphohistiocytic meningoencephalitis appeared with peculiar clinical signs (spastic gait, stiff tail), the onset of the disease was late (mean 9 years) and the progression of signs was very slow (mean 11 months). A causative agent could not be identified. Thus, the cats in this study had been affected by a unique, previously unreported condition (De Risio et al., 2012). MRI images of the brain must be evaluated carefully as many mass lesions regarded as tumors can be fungal or protozoal granulomas (Foster et al., 2001; Pfohl and Dewey, 2005). Brain abscesses due to bite wound were also identified in cats (Costanzo et al., 2011).

Ischemic cerebrovascular accidents rarely appear in cats and they are usually associated with concurrent disease (Whittaker et al., 2018).The prognosis is guarded, although this will be dependent on the severity and type of the relevant concurrent disease (Whittaker et al., 2018).

Head trauma is common in dogs and cats and typically results from kicks, bites, motor vehicle or missile object injuries (Braund, 2003). A relevant previous study indicated that brain injuries in cats were typically caused by crash accidents (Syring et al., 2001). Traumatic brain injury reportedly causes structural

epilepsy in companion animals (Bailey and Dewey, 2009; Braund, 2003; Parent and Quesnel, 1996). Although epileptic seizures that appear as a result of traumatic brain injury are often refractory to antiepileptic treatment in humans (Herman, 2002), cats with medical history of mild to moderate head trauma had ≤ 5.6% probability of developing post-traumatic epileptic seizures (Grohmann et al., 2012). However, clinicians are advised to monitor cats with history of head trauma for development of structural epilepsy (Grohmann et al., 2012).

The most common central nervous system neoplasm in cats is meningioma (Kline, 1998; Matoon and Wisner, 2004; Pakozdy et al., 2010). It can occur singly or in multiple sites (Kline 1998). Meningiomas typically affect the older feline patient (>8 years old), males are disproportionally affected over females (Gorden et al., 1996; Kornegay, 1991). Another neoplastic cause of primary or secondary central nervous system neoplasm is lymphoma (Kline, 1998). The signalment of cats with lymphoma differs from than with meningioma, in that these cats tend to be young to middle-aged (between 7-10 years old) (Kline, 1998).

Cats may develop obstructive hydrocephalus secondary to infectious diseases such as feline infectious peritonitis (FIP) (Lavely, 2014). Hydrocephalus can result from autosomal recessive inheritance in Siamese cats (Hoskins, 1990).

Structural epilepsy was more frequent than idiopathic epilepsy in cats (Pakozdy et al., 2010). Idiopathic epilepsy reflects conditions that have no underlying cause (Bailey and Dewey, 2009) and genetic mechanisms are the presumed etiology (Cunningham and Farnbach, 1987). Cats determined as having a seizure disorder with unidentified etiology are referred as idiopathic epileptics (Bailey and Dewey, 2009).

Idiopathic epilepsy is recognized in cats; however, cats are generally older at the onset of seizures than are dogs with idiopathic epilepsy (Bailey et al., 2008). Although idiopathic epilepsy was thought to be rare in cats (Bailey and Dewey, 2009; Berg and Scheffer, 2011;Kline, 1998;Pakozdy et al., 2010;Schrieflet al., 2008), new evidence suggest that between 21% and 59% of cases are idiopathic (Cizinauskas et al., 2011; Quesnel et al., 1997b; Schriefl et al., 2008), another study indicate a 25% of cats with seizures as having idiopathic epilepsy (Schriefl et al., 2008). Other authors have defined idiopathic epilepsy as inherited epilepsy (Parent and Quesnel, 1996; Quesnel et al., 1997b; Shell, 2000).

In contrast to idiopathic epilepsy in dogs, whose diagnosis was based on age and normal clinicopathological testing, this should not be applied in cats (Schriefl et al, 2008). Additionally, idiopathic epilepsy in dogs is assumed to be genetic in origin but no information support such an assumption in cats (Pakozdy et al., 2010).

In many cases of feline epilepsy, an underlying cause of epileptic seizures was suspected but never proven ante-mortem (Kline, 1998). These include previous post-traumatic, post-inflammatory and post-ischemic lesions that were quiescent and non-progressive (Kline, 1998). This form of idiopathic epilepsy was probably more common than true idiopathic epilepsy in the cat (Parent and Quesnel, 1996).

## CLINICAL SIGNS

Epileptic seizures can have a wide range of clinical signs and are not necessarily typical in all cases (Pakozdy et al., 2014). Seizure episodes can be generalized, with tonic-clonic movements as observed in dogs, focal, or focal evolving to generalized epileptic seizure (Bailey and Dewey, 2009; Berendt et al., 2015; Kline, 1998). Cats with focal seizures will twitch the eyelids, whiskers and/or ears either in combination or separately. Head shaking may occur as well as jerking of the body. They may salivate, urinate and their pupils may transiently dilate. Simultaneously, they may vocalize continuously and they may experience a temperature increase caused by hyperthermia (Kline, 1998). Focal episodes are frequently isolated or have a secondary generalization (Bailey and Dewey, 2009; Berendt et al., 2015). Focal continuous epileptic seizures occur more often as a presentation of status epilepticus in cats than in dogs (Kline, 1998).

Etiology was not associated with the type of seizure (Schriefl et al., 2008; Tokem et al., 2006). Idiopathic, generalized seizures are uncommonly observed in feline patients while partial and complex partial seizures prevail (Parent and Quesnel, 1996). Historically, focal epileptic seizures have been associated with structural lesion of the forebrain (Parent and Quesnel, 1996; Quesnel et al., 1997b), although the presence of focal lesions does not rule out a diagnosis of idiopathic epilepsy (Bailey and Dewey, 2009; Quesnel et al., 1997b; Schriefl et al., 2008). Tokem (2006) found that generalized epileptic seizures are the most common

seizure pattern in cats with intracranial neoplasia. In feline idiopathic epilepsy, focal and generalized epileptic seizures occur with relatively equal frequency (Schriefl et al., 2008). Focal epileptic seizures occur with near equal frequency in cats with idiopathic and cats with structural epilepsy (Pakozdy et al., 2010; Quesnel et al., 1997b; Schriefl et al., 2008). The frequency of intracranial disease as the most common underlying cause of epileptic seizures in cats is controversial (Barnes et al., 2004; Finnerty et al., 2014; Quesnel et al., 1997a; Schriefl at el., 2008).

Furthermore, a new type of seizure in cats (audiogenic reflex seizures) have been described (Lawrie et al., 2016). This type of seizure was precipitated by sensory stimuli and especially by loud sounds (Lawrie et al., 2016). Clinically, they can appear as generalized tonic-clonic seizures, myoclonic jerks, clinical absences or presumed absence seizures (Lawrie et al., 2016).

Timmann (2008) found that the occurrence of epileptic seizures in FIP cats indicated extensive brain damage and can, therefore, be considered to be an unfavorable prognostic sign.

It was presumed that lunar cycle can affect the frequency of epileptic seizures however Browand-Stainback (2011) reported no difference in seizure occurrence in the different phases of lunar cycle in both dogs and cats.

## DIAGNOSIS

The diagnostic work-up for an epileptic cat include a thorough history, clinical and neurological examination, clinicopathological evaluation and advanced imaging (Bailey and Dewey, 2009). The baseline laboratory investigation includes a complete blood count (CBC), serum biochemistry profile, thyroid profile, blood pressure monitoring, measurement of serum bile acids concentration and urinalysis (Bailey and Dewey, 2009). These non-invasive tests may help to diagnose reactive seizures and are useful in planning anesthesia for any advanced imaging (Bailey and Dewey, 2009).

A complete funduscopic examination is imperative in all cases (Gunn-Moore and Reed, 2011). Advanced diagnostics include magnetic resonance imaging (MRI) or computed tomography (CT) and potentially cerebrospinal fluid (CSF) analysis (Bailey and Dewey, 2009). However even in high-field MRI, small cerebrovascular or inflammatory lesions may not be visible and thus whether the etiology cannot be identified by the diagnostic work-up it does not necessary mean that epilepsy is idiopathic (Pakozdy et al., 2010). A CSF tap is indicated if the imaging is normal or suggestive of intracranial disease and the cat is believed to have normal intracranial pressure (Bailey and Dewey, 2009). If there is a space-occupying mass suspected or there is evidence of brain herniation, a CSF tap is contraindicated (Bailey and Dewey, 2009). Normal CSF is clear and colorless with fewer than 5 cells/ μl and less than 27mg/ dl protein (for a cisterna magna tap) (Bailey and Dewey, 2009). Abnormalities in CSF are very sensitive indicators of intracranial disease but they are not specific (Bailey and Dewey, 2009). However, when evaluated with CT/MRI, CSF analysis can be a helpful diagnostic tool (Bailey and Dewey, 2009). Bacterial cultures and infectious diseases titers *(Cryptococcus, toxoplasmosis* and *FIP*) may also be useful tests to perform on CSF (Dewey, 2006; Foster et al., 2001; Pfohl and Dewey, 2005; Timmann et al., 2008).

## INDICATIONS TO START ANTIEPILEPTIC THERAPY

There is no consensus among veterinary neurologists about when antiepileptic treatment should be started (Pakozdy et al., 2014). It was suggested not to start antiepileptic treatment after a single epileptic seizure, but the occurrence of frequent epileptic seizures over a short period of time warrants treatment commencement (Platt, 2001). Others recommended aggressive treatment for cats after a few seizure episodes (Quesnel et al., 1997a). An aggressive early start of treatment can be beneficial as the cat could avoid cluster seizures and refractory epilepsy (Pakozdy et al., 2014). The decision to start the treatment should be taken on a case-by-case basis after considering the severity of epileptic seizure, ictal signs, risk of treatment, owner compliance, serum monitoring possibilities, and the difficulties with long-term oral application (Pakozdy et al., 2014). Maintenance therapy is specifically recommended when a cat presents with more than one seizure within 6 months (either generalized or focal), has more than one cluster event (defined as more than one seizure in a 24 h period), or experiences status epilepticus (ie, continuous seizure activity that lasts more than 5 minutes or the presence of multiple seizures without returning to normal in between the seizures) (Bailey and Dewey, 2009). Early AED administration in cats with suspected idiopathic epilepsy has been shown to lower the seizure

frequency within the first year of therapy (Pakozdy et al., 2012).

Therapy with antiepileptic drugs is recommended when epileptic seizures occur post-trauma (Bailey and Dewey, 2009). Grohmann (2012) found that cats with medical history of mild to moderate head trauma had ≤ 5.6% probability of developing post-traumatic seizures. In another study, it was mentioned that the seizure could appear immediate after head trauma or delayed and thus the owners should be informed about the potential need for antiepileptic drug either at the time of the trauma or in the future (Kline, 1998).

It is strongly advised to treat cats with antiepileptic drugs when there is evidence of structural forebrain disease (obtained via MRI/CT, CSF analysis) such as neoplasia, infectious or non-infectious encephalitis, or congenital disease. Additional therapy is also warranted to treat the primary cause of the seizures; however, even after appropriate management (ie, surgery to remove a meningioma), AED administration is often necessary and is typically lifelong (Bailey and Dewey, 2009).

## ANTIEPILEPTIC TREATMENT

Phenobarbital (PB) is the current drug of choice in cats with multiple seizure episodes (Berg et al., 2006; Dewey, 2006; Finnerty et al., 2014; Thomas and Dewey, 2008). It is available in both oral and intravenous formulations (Bailey and Dewey, 2009). Phenobarbital is relatively affordable and historically has a low incidence of adverse effects, thus PB is an excellent AED choice for cats (Parent and Quesnel, 1996; Platt, 2001;Schwartz-Porsche and Kaiser, 1989).There is one report in which adverse effect of PB in a cat is mentioned; it included depression, anorexia, cutaneous eruptions and severe generalized lymphadenopathy, the signs appeared 21 days after PB administration and resolved 7-14 days after PB discontinuation (Ducote et al., 1999). There are several studies supporting the efficacy of PB of seizure control in epileptic cats (Finnerty et al., 2014). Seizure control was achieved in most cats with serum PB concentrations between 15-45 μg/ml, regardless of the cause of the seizures (Finnerty et al., 2014).

There are few anecdotal reports on levetiracetam as an antiepileptic drug in the clinical setting (Bailey and Dewey, 2009). Levetiracetam was used as an adjunctive anticonvulsant therapy in cats with idiopathic epilepsy (Bailey et al., 2008). There was a marked reduction in seizure frequency (>50% reduction) and in some cases there were no seizures after levetiracetam initiation (Bailey et al., 2008). Levetiracetam is well tolerated in cats and may be useful as an adjunct to phenobarbital in cats with idiopathic epilepsy (Bailey et al., 2008). Levetiracetam proved to be a promising option as a sole antiepileptic treatment in cats with seizure disorders. Despite its association with the honeymoon period in dogs and people (brief decrease in the number of seizures, then return to a frequency similar to what occurred prior to levetiracetam initiation), it did not seem to desensitize cats for over a year after its first administration. (Bailey et al., 2008; Kinirons et al., 2006; Volk et al., 2007).

Although, oral diazepam has a longer elimination time in cats (15-20h) than in dogs (3-4h), and the cats do not appear to develop a functional tolerance to the drug, it has been associated with a potentially fatal idiosyncratic hepatotoxicosis (Center et al., 1996; Hughes et al., 1996).

Bromide has been associated with life-threatening idiosyncratic allergic pneumonitis, reported in 35-42% of cats (Boothe et al., 2002; Wagner, 2001). Further studies report that 40% of cats receiving potassium bromide developed moderate to severe bronchoalveolitis/ pulmonary fibrosis and some of those cats were euthanized due to severity of clinical signs (Folger, 2009).

Gabapentin is another potential AED (Thomas and Dewey, 2008) however, there are no published data regarding chronic gabapentin therapy in feline epilepsy (Pakozdy et al., 2012).

Most cats can improve their quality of life and reduce aggression through a successful control (>50% reduction of seizures) regardless of etiology. Although there are reports showing a long term survival time in epileptic cats (range 3-21 months) (Quesnel et al., 1997a), some others disagree (Barnes et al., 2004; Schriefl et al., 2008). Survival time was shorter in cats with structural epilepsy; however in the same study survival time was longer in cats with probable structural epilepsy (epilepsy without any extracranial or identified intracranial disease that is not suspected to be genetic in origin) (Barnes et al., 2004), indicating that the cause and the degree of the brain damage in structural epilepsy can influence survival time. Euthanasia was elected soon after a diagnosis was established due to poor prognosis (Barnes et al., 2004) in cats. Poor seizure control, despite appropriate AED

administration was characterized as refractory epilepsy (Munana, 2013). Refractory epilepsy occurs in cats, less frequently than in dogs (Munana, 2013).

## PROGNOSIS

Status epilepticus is a poor prognostic indicator in canine and feline epilepsy (Bateman and Parent, 1999; Schriefl et al., 2008). On the contrary, in epileptic dogs, the type of seizures was not associated with the survival time (Berendt et al., 2007).

Not only the number but also the severity of epileptic seizures is crucial (Pakozdy et al., 2012). It is possible that even a single epileptic seizure per year is harmful for patients because of its severity (status epilepticus, cluster seizures, prolonged post-ictal period); while other patients may not be affected by experiencing one epileptic seizure a week (Berg and Scheffer, 2011). For this reason it is important that not only the number of epileptic seizures, but also the adverse effects and the patient's quality of life are taken into account when evaluating control of epileptic seizures and effects of treatment, although naturally quality of life is a highly subjective variable (Pakozdy et al., 2012). The complete remission of epileptic seizures in cats is rare (Pakozdy et al., 2012) and most cats should be maintained on anti-epileptic therapy (Platt, 2001).

## CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

# Effect of a nanocomposite containing ostrich eggshell on calvarium healing in the rabbit: a pathologic study

**H. Alemi[1], A. Asghari[1*], G. Abedi[1], A. Akbarzadeh[2,3], P. Mortazavi[4]**

[1] *Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

[3] *Universal Scientific Education and Research Network (USERN), Tabriz, Iran*

[4] *Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

**ABSTRACT.** The aim of the current study was to determine effect of a nanocomposite containing ostrich eggshell (NCOE) on the calvarium healing in the rabbit. Fresh ostrich eggshell was ground (300-500 µm), treated in phosphate-containing solutions and sterilized by gamma irradiation. Fifteen New Zealand white adult male rabbits were used. Four full-thickness skull defects were created in the calvarium. The first defect kept unfilled (control). The second defect was filled with autograft bone. The third defect was filled using NCOE. The fourth defect was filled with mixture of the autograft+NCOE bone. At 30, 60 and 90 days after surgery animals were euthanized and tissue specimens were collected and stained with hematoxylin eosin and trichrome staining method. Microsections were examined to assess the extent and intensity of inflammation, calvarium formation status and foreign body reaction. According to the results, filling defect significantly increased in NCOE-treated rabbits compared to the control group at 30 and 60 days post-surgery ($P<0.05$). There a statistically significant difference between experimental groups compared to the control group at 30 and 60 days post-surgery ($P<0.05$) while no statistically significant differences were observed among autograft, NCOE, autograft+NCOE ($P>0.05$). Also, absorb material significantly decreased in NCOE and autograft+NCOE groups compared to the control group at 60 days post-surgery ($P<0.05$). The filling defect significantly increased in autograft, NCOE and NCOE+autograft groups compared to the control group at 90 days post-surgery ($P<0.05$). There was no significant difference on inflammation and absorb material among the groups at 90 days post-surgery ($P>0.05$). These results suggested NCOE+autograft has improved the rate of calvarium healing in rabbits.

***Keywords:*** Nano- composite, Ostrich eggshell, Calvarium autograft, Rabbit

*Corresponding Author:*
A. Asghari, Department of Clinical Science, Science and Research Branch, Islamic Azad University, Tehran, Iran
E-mail address: dr.ahmad.asghari@gmail.com

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## INTRODUCTION

In the last decade, guided tissue regeneration provided new research area in the bone reconstruction field (Lim et al., 2010). One of the most important factors is biocompatibility and degradation rate of the membranes (Lim et al., 2010). Autogenous bone grafts (ABG) are known as the gold standard and preferred augmentation material (Jones et al., 2010). The ABG has high compatibility with the host tissue and effective in bone graft healing without immune response (Lindhe et al. 2008). High costs donor and prolonged operation times are the disadvantages of the ABG (Jones et al., 2010). Combination of the bone grafts with other treatments enhances the engraftment, bone formation and defect healing (Marx, 2004). Application of the adjuvant bone graft as primary goal of guided bone regeneration (GBR) is an alternative method to promote bone regeneration without additional bone grafting (Yang et al., 2014). Several synthetic allografts have been used for bone regeneration, but there is growing interest to use natural allografts (Toker et al., 2012).

Avian egg shell contains calcium carbonate (97.4%), magnesium phosphate (1.9%) and triphosphate (0.7%) (Durmuş et al., 2008), however, the composition differs among species. The high mineral level can accelerate hydroxyapatite formation in bone regeneration (Leung et al., 2005). Also, eggshell matrix contains proteins (70%), Chondroitin sulphates A and B (35%) and polysaccharides (11%) (Dupoirieux et al., 1995). Dupoirieux et al., (2000) studied role of the pericranium and eggshell as space fillers used in combination with GBR in rat and no resorption or osteoconduction was revealed in eggshell powder of the hen eggshell had safe and inexpensive application in rabbit bone defect model (Dupoirieux et al., 1995). Application of the ostrich eggshell powder had no effect on bone regeneration in rabbits (Durmuş et al., 2003).

Large ostrich grafts are suitable as onlay graft, however a complementary osteosynthesis is suggested to improve osteointegration (Dupoirieux et al., 2001). Eggshell powder increased cell growth and tibia remodeling in dogs (Yadegari et al., 2015). Nanocomposites or nanomaterials contain high level of the collagen and hydroxyapatite and their application has drawn attention compared to bone grafts. There is scarce information on effects of the nanomaterials in bone formation. The small molecular structure of the nanocomposites increases the bioactivity of the materials used for bone defect healing and regeneration (Dupoirieux et al., 2001). This is due to composition and structural similarity with natural bone as well as larger surface area and superior mechanical strength (Biazar et al., 2015). Because of complexity of the osteoclast and osteoblasts and the other factors the clinicians were compelled to search for alternative bone graft substitutes in regenerating process in human (Dupoirieux et al., 2001). In a recent study, Alemi et al. (2018) showed that nanocomposite containing ostrich eggshell (NCOE) + autograft has positive effects on bone density without adverse effect on white and red blood cells hemoglobin, hematocrit, mean cell volume and platelets levels in rabbit. There is no information on role of the NCOE in bone formation in the rabbit. The aim of the current study was to determine effect of the NCOE on calvarium healing in rabbits.

## MATERIALS AND METHODS

### Animals

Fifteen adult male New Zealand white rabbits (3-3.5 kg) were purchased from the Razi Vaccine and Serum Research Institute (Tehran, Iran) (Durmuş et al., 2008; Paknejad et al., 2015). For acclimatization the animals were kept in individual cages in the laboratory at constant and optimum environmental and nutritional conditions (22 ºC, relative humidity 50%) with a 12-hour light/dark cycle. During the study animals provided *ad libitum* commercial chew pellet and tap water. Study procedures were done during the 10:00-17:00 h light phase and executed in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann, 1983) and National Institutes of Health (USA) and the current laws of the Iranian government. All experiment procedures were approved based on the guidelines for the animal care board of the Islamic Azad University, Faculty of Veterinary Medicine and besides, the current study was approved by the university ethics committee (Ethic code:25876).

### Ostrich eggshell preparation

Fragmented ostrich eggshells were immersed in boiling sterile distilled water and the outer and inner shell membranes of the eggs were removed with forceps. The shells were crushed and sieved until particles (particle diameter, 1mm; porosity, 75%) were obtained (Uraz et al., 2013). Ostrich eggshell ground the into 300-500 μm pieces using an electrical mill, washed 3 times with distilled water, dried and steril-

ized using ethylene oxide (Durmuş et al., 2003). The eggshell powder was immersed in sodium hypochlorite (5%) and then organic components were removed by sodium hydroxide solution (Durmuş et al., 2003). Eggshell powder subsequently washed in deionized water and heat-treated at 300 ºC for 24 h. Then treated in phosphate-containing solutions with different hydrothermal conditions (Pastoureau et al., 2010). Soaked in sealed glass bottles containing phosphate buffer saline at 80 ºC for 6 days (the solution replaced every 3 days) (Permuy et al., 2014), then soaked in Teflon lined reactor containing 2 wt % di-ammonium phosphate solution at 150 ºC for 24 h, then transferred in a phosphate-containing solution (19.5 mM PO43, 30 mM Naþ and 4.3 mM Kþ) at 80 ºC for 6 days (Permuy et al., 2014). All ostrich eggshell particles were sterilized by gamma irradiation before use (Permuy et al., 2014).

**Preparation of nanocomposite containing ostrich eggshell**

The composite was prepared from monomer loop polymerization in molten state and presence of a tin octoate catalyst. Capro-lactone with a molecular weight of 1000 was accurately weighed and placed in 3 span balloon equipped with Nitrogen gas inlet and outlet and heated by magnetic stirrer equipped. After melting the PEG, the molten catalysts of tin octoate (0.05 wt % of raw materials), added to begin the polymerization reaction and continued with gentle stirring and nitrogen gas flow. At the end of the polymerization, the solution was cooled to room temperature. Solid polymer was dissolved in dichloromethane and poured into a large volume of dry ethyl ether. The polymer was dried and solvent removed by a vacuum attached to the desiccator. Polycaprolactone nanocomposite-ostrich egg shell (PCL-HA) was prepared by particle flush and freeze drying (Park et al., 2008).

**Sample characterization**

The characteristics of the prepared samples (NCOE) were evaluated by scanning electron microscope (SEM), powder X-ray diffractometry (XRD, X'Pert-APD; Philips, Netherlands) (Park et al., 2008).

**Surgical Protocol**

Six hours prior the initiation of the study, animals were food deprived and 1 hour before surgery fasted from drinking. Then animals were anesthetized with an intramuscular (i.m) injection of ketamine hydrochloride 10% (Alafason, Woeden, Holland, 40mg/kg) and 2% xylazine (Alafason, Woeden, Holland, 5mg/kg) and then were placed in sternal recumbency position on the operating table. The head of the rabbit was shaved and scalp prepared with povidone-iodine solution (Betoni-Junior et al., 2013). A longitudinal anteroposterior incision (10 cm) was made along the midline of the skull from the midpoint of the base of ears using No. 15 surgical blade. Before incising the periosteum, the skin was retracted by a surgical mosquito and then using a periosteal elevator periosteum was separated from the bone surface cranial to caudal. Four bone defects (internal 8 mm diameter) were created in the calvaria (Takauti et al., 2014). Defects were created on both sides of the sagittal suture without crossing the midline using electric 2000 rpm hand piece and 8 mm diameter milling round surgical trephine. To prevent overheating until holes reached the meningeal membrane (the soft meningeal membrane was palpable) 0.9% physiologic saline solution was used (Betoni-Junior et al., 2013). The first defect was maintained unfilled and kept as control. The second defect was filled with autograft bone derived from the site of the defect (Takauti et al., 2014). The third defect was filled using NCOE. The fourth defect was filled with mixture of the autograft + NCOE bone. The filling and placement of the material into the pits was done in a counter clockwise direction and without pressure to ensure the particles did not enter the meningeal space (Betoni-Junior et al., 2013). After placing the materials, the periosteum was sutured with 4/0 simple absorbable sutures (Polyglycolate coated, SUPA, Iran). The calvarium was sutured with 3/0 nylon sutures and skin was sutured with a single simple suture (Monofilament Polyamide, SUPA, Iran). After animal was coming out of anesthesia, they were transferred to a warm place until regaining full consciousness and then into cage. To prevent infection and relieve pain, day post-operative, cefazolin 1g, Exir, Iran (20 mg/kg; i.m) and tramadol 50 mg, Exir, Iran (20 mg/kg; i.m) were injected. If swelling or inflammation appeared in the surgery area, the sutures were removed and the presence of infection or discharge was evaluated but there were no complications in current study. Skin sutures were removed 10 days after surgery (Betoni-Junior et al., 2013).

**Assessment of bone regrowth**

At 30, 60 and 90 days after surgery, 5 animals were euthanized with pentobarbital (88mg/kg IV) and tissue specimens were collected in 10% for neutral buffered formalin solution to evaluate regrowth. Af-

ter fixation, tissues were decalcified in 5% nitric acid. Then 5-micron sections were cut using a microtome (Leica RM 2145 Rotary, Germany) for processing and embedding of tissue. Then samples were stained with hematoxylin eosin [H&E] and trichrome staining method (Jörundsson et al. 1999). Microsections were examined with light microscope (Olympus, CX21i, Germany) and MOTIC camera and image analyser software version 1.6.0 to assess the extent and intensity of inflammation, formation status and foreign body reaction. For each section 10 microscopic fields were evaluated.

**Evaluation of the formation**

Inflammation was graded using a five-tiered grading system as follows: grade (0): no inflammatory mononuclear cells, (grade I): mild inflammation with mononuclear inflammatory cells (<25%), grade (II): presence of the focal mononuclear inflammatory cells (25-50%), grade (III): focal inflammation with presence of the mononuclear inflammatory cells (50-75%) and grade (IV): focal inflammation with presence of the high level of the mononuclear inflammatory cells (>75%). The filling of the defect determined by investigation of the newly formed bone trabecula inside the cannula using a nine-tried grading system as follows: (0): not filling, (I): just fibrous and low cartilage, (II) same percent of fibrous and cartilage, (III) low fibrous and high cartilage, (IV) just cartilage, (V) high cartilage and immature bone, (VI):same percent of cartilage and immature bone, (VII): low cartilage and high level of immature bone, (VIII): healed with immature bone and (VIIII): healed with mature bone. The remodeling was determined using a five-tiered score as follows: (0): not remodeling, (I): <25% remodeling, (II): 25-50% remodeling, (III): 50-75% remodeling and (IV): >75% remodeling. The material absorbance was determined using a 4 grading system as follows: (grade 0): not absorbed, (grade I): 25-50%, (grade II): 50-75% and (grade III) fully absorbed (Huo et al. 1991).

**Statistical analysis**

The parametric data analyzed with one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean values ± standard error of mean (SEM). Where heterogenecity occurred, the groups were separated using Duncan Multiple Range Test. The KruskaleWallis test was used to compare group medians for histopathological scores. $P<0.05$ was considered to denote significant differences between groups.

**RESULTS**

The SEM image of the NCOE is presented in figure 1. The X-ray diffraction pattern of the NCOE is presented in figure 2. The NOCE showed different surface morphology platelet-like, needle-like, or rod-like microstructure.

Effects of NCOE on filling defect, inflammation, remodeling and absorb material on calvaria at 30 days post-surgery is presented in figure 3. Filling defect statistically increased in NCOE-treated rabbits compared to the control group at 30 days post-surgery ($P<0.05$). There were significant differences between experimental groups compared to the control group at 30 days post-surgery ($P<0.05$) while no statistical differences observed among autograft, NCOE, autograft + NCOE ($P>0.05$). The absorbed material significantly decreased in experimental groups compared to control group. No statistically significant differences observed between NCOE and autograft + NCOE ($P>0.05$).

Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria at 60 days post-surgery is presented in figure 4. Significant increase was observed on filling defect in NCOE compared to the control group ($P<0.05$). Significant increase observed on inflammation in experimental groups compared to the control group ($P<0.05$) while no statistical differences observed among autograft, NCOE, autograft + NCOE ($P>0.05$). Also, absorb material significantly decreased in NCOE and autograft + NCOE groups compared to the to the control group at 60 days post-surgery ($P<0.05$).

The filling defect significantly increased in autograft, NCOE and NCOE + autograft groups compared to the control group at 90 days post-surgery ($P<0.05$). There was no statistical significant difference on inflammation and absorb material among the groups at 90 days post-surgery (figure 5) ($P>0.05$). The histological images of the tissue at 30, 60 and 90 days post-surgery are presented in figures 6-17.

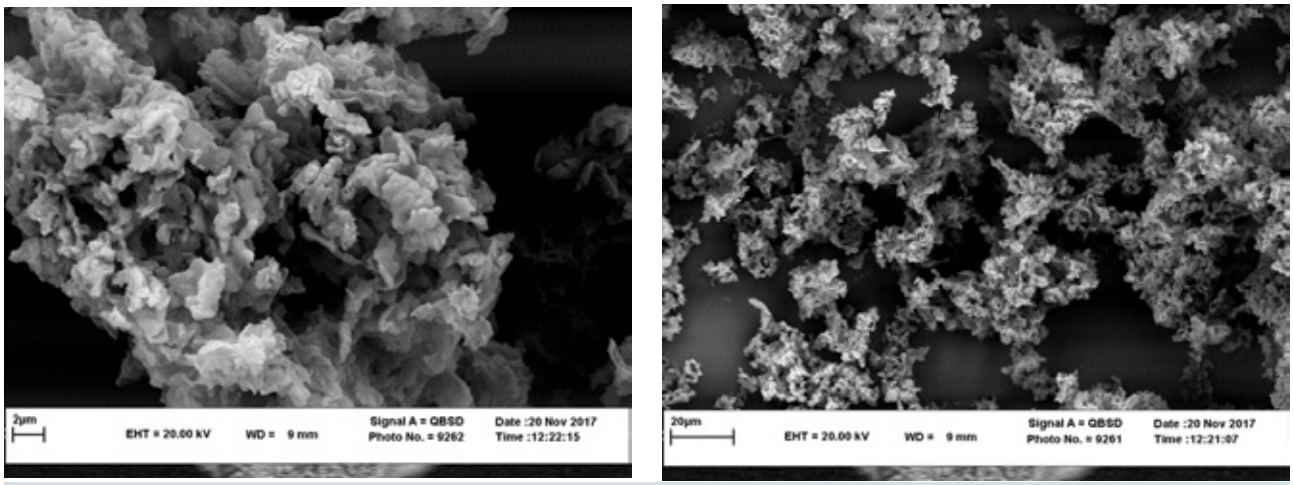


**Figure 1.** The electron microscope image of the ostrich Eggshell nanocomposite

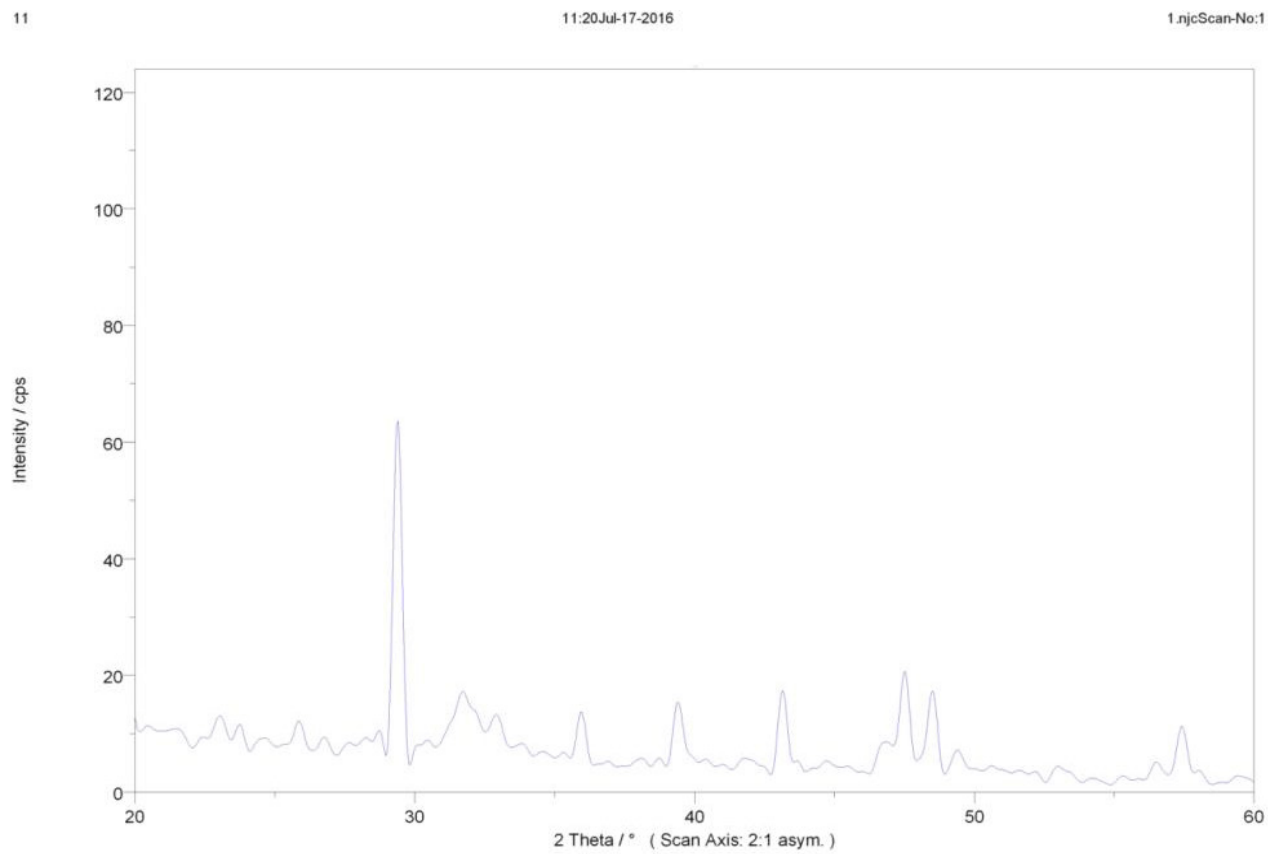


**Figure 2.** X-ray diffraction patterns of the NCOE. Arrows indicate the peaks of hydroxyapatite

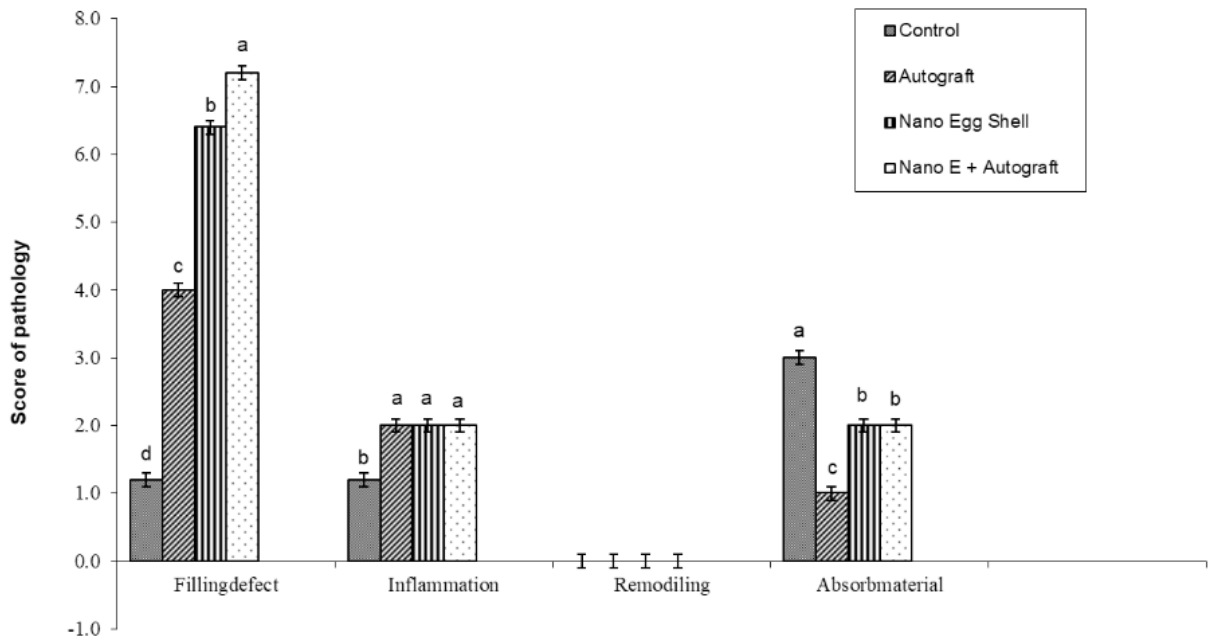


**Figure 3.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 30 days post-surgery (P<0.05). NCOE: nanocomposite containing ostrich eggshell

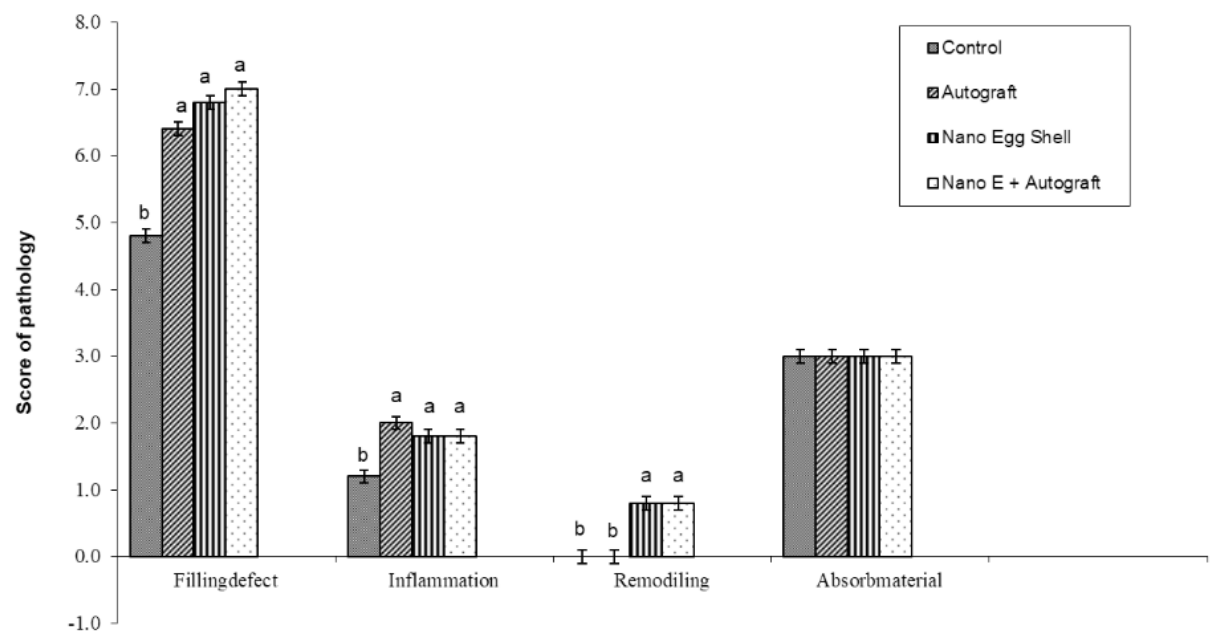


**Figure 4.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 60 days post-surgery (P<0.05). NCOE: nanocomposite containing ostrich eggshell

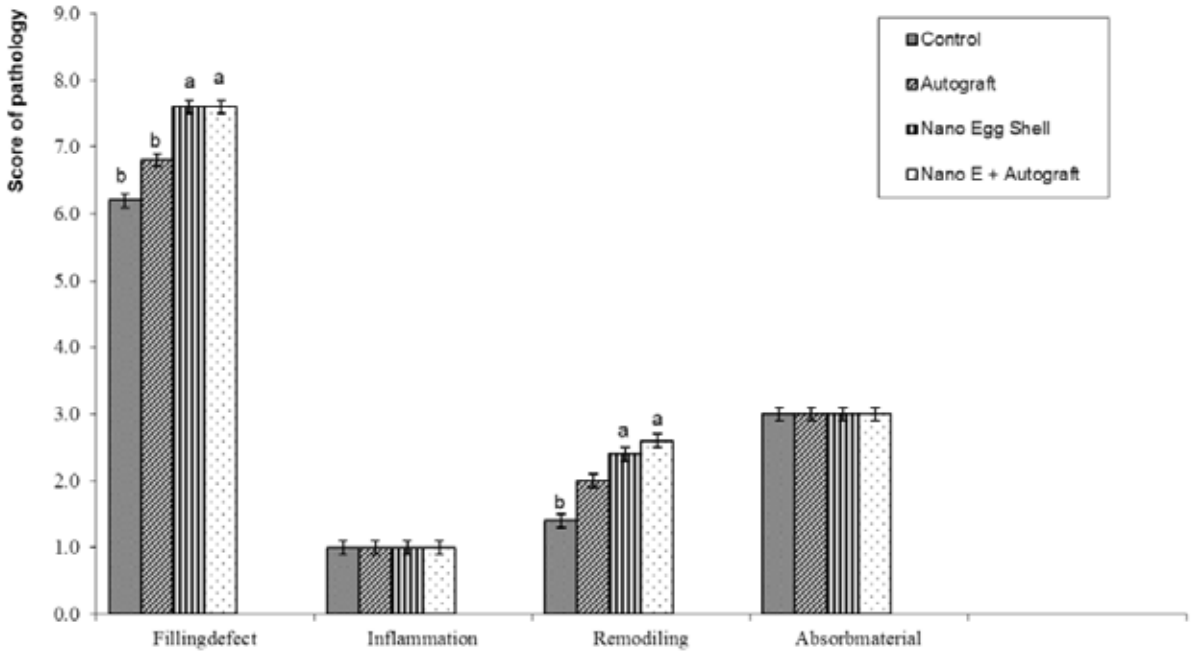


**Figure 5.** Effects of the NCOE on filling defect, inflammation, remodeling and absorb material on calvaria bone at 90 days post-surgery ($P<0.05$). NCOE: nanocomposite containing ostrich eggshell

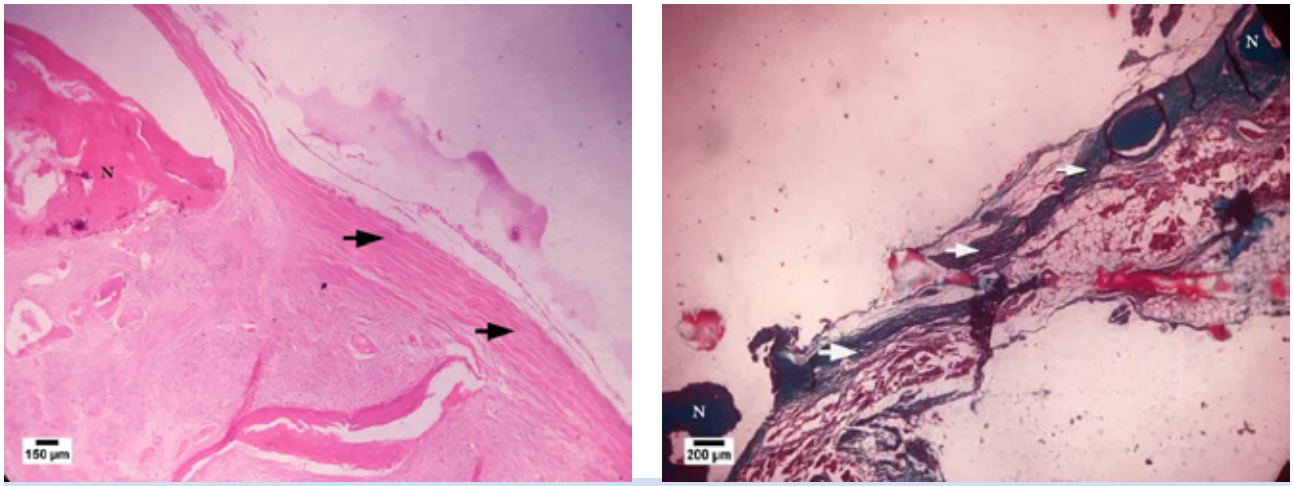


**Figure 6.** Control group (30 day) that is filled with a large amount of fibrous tissue (arrows). A part of the normal calvarium bone (N) is seen. Control group (30 day) that is filled with a large amount of fibrous tissue (arrows). A part of the normal calvarium bone (N)is seen (left: H&E, Right: Masson trichrome, 40×)

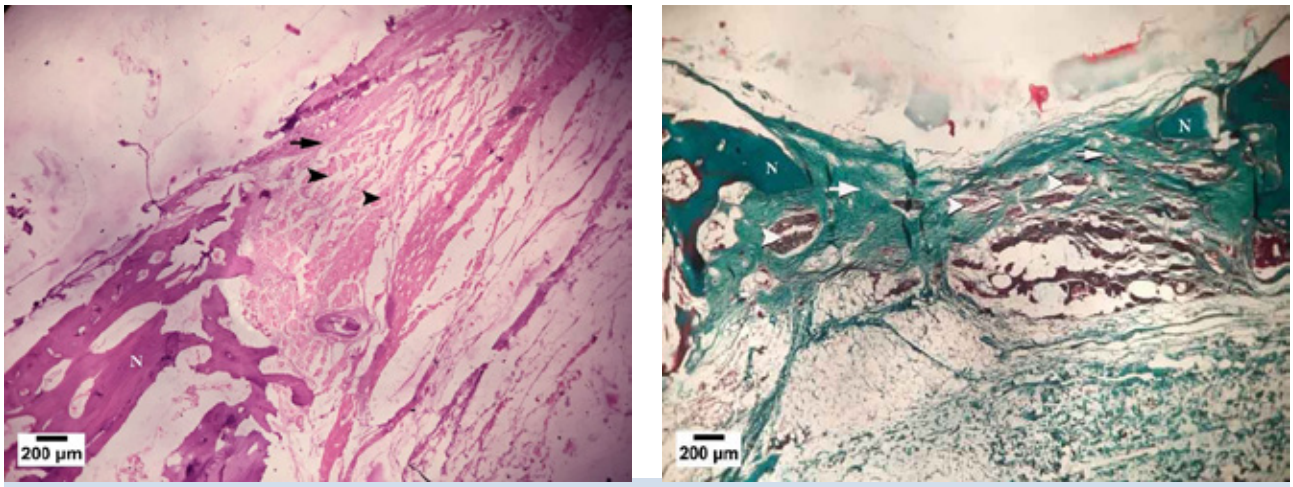


**Figure 7.** Autograft group (30-day) with a large amount of cartilage (arrow head) and a few fibrous tissue (arrows) that filled defect. A part of the normal bone (N) is seen. Autograft group (30-day) with a large amount of cartilage (arrow head) and a few fibrous tissue (arrows) that filled defect. A part of the normal bone (N) is seen (left: H&E, Right: Masson trichrome, 40×)

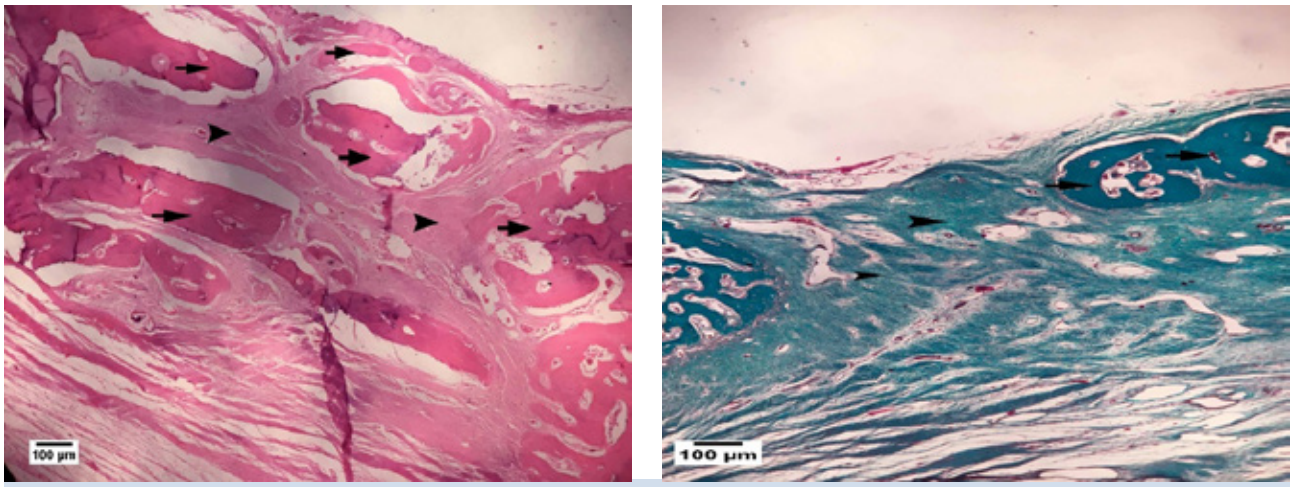


**Figure 8.** Nano group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow).Nano group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

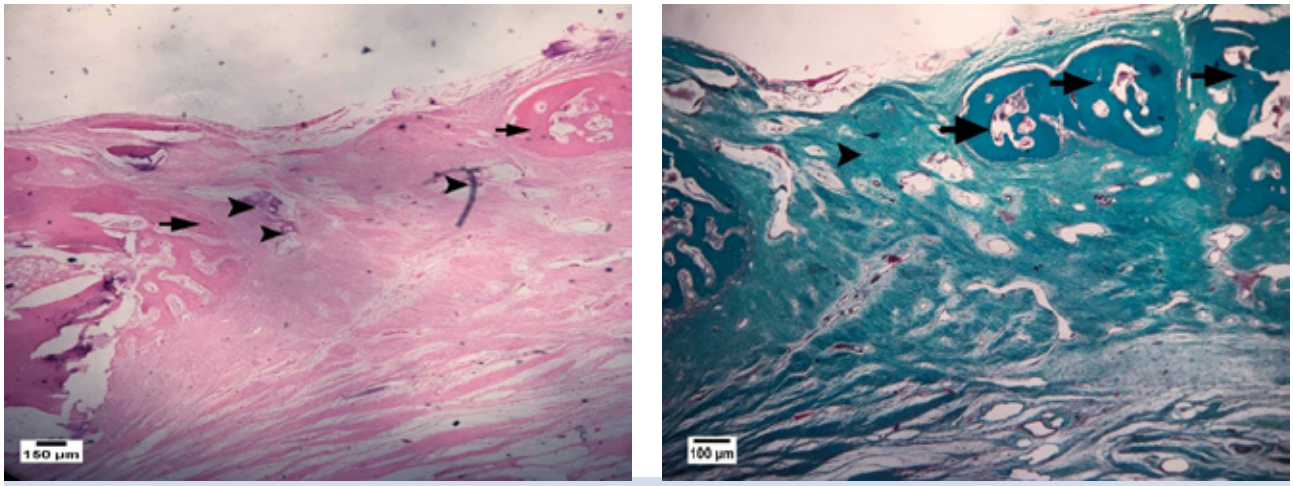


**Figure 9.** Nano autograft group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow). Nano autograft group (30-day) that the defect filled with a large amount of immature bone tissue (arrow head) and a few amount of cartilage tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

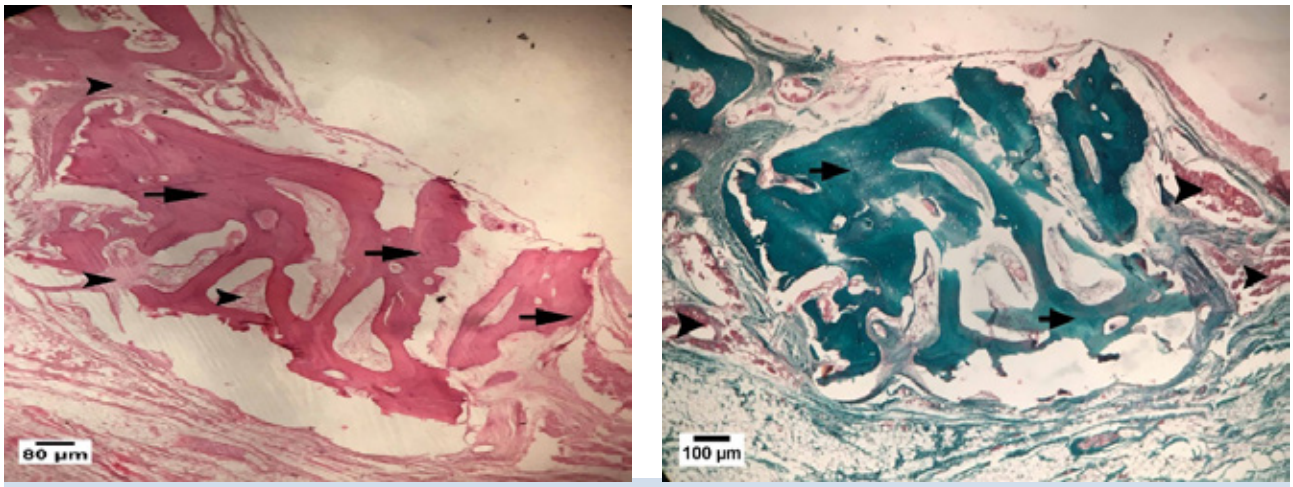


**Figure 10.** Control group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and immature bone tissue (arrow). Control group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 100×)

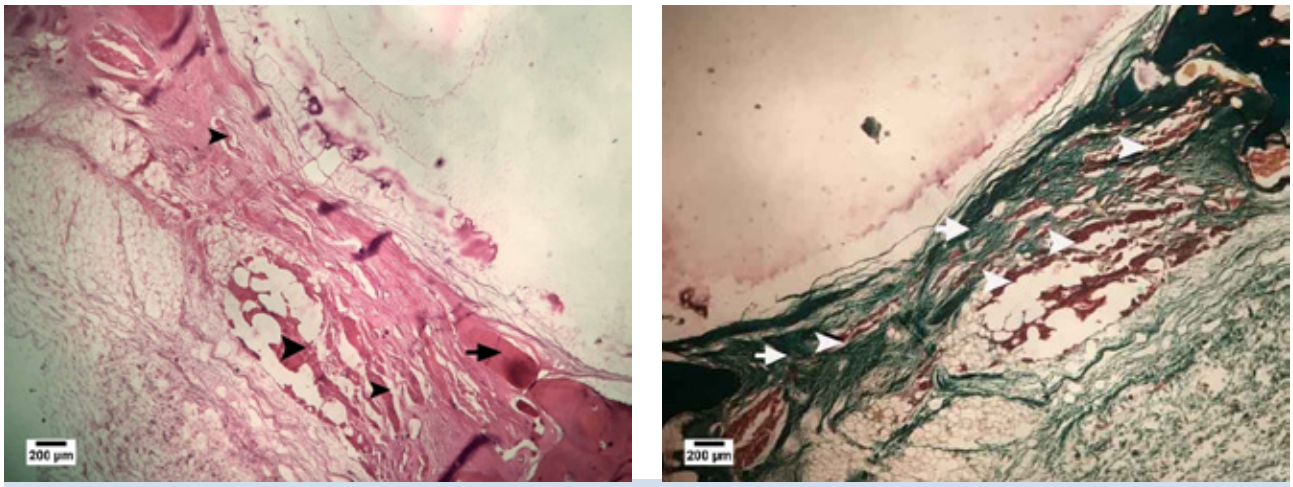


**Figure 11.** Autograft group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and few immature bone tissues (arrow). Autograft group (60-day) that the defect filled with a large amount of cartilage tissue (arrow head) and few immature bone tissues (arrow) (left: H&E, Right: Masson trichrome, 40×)

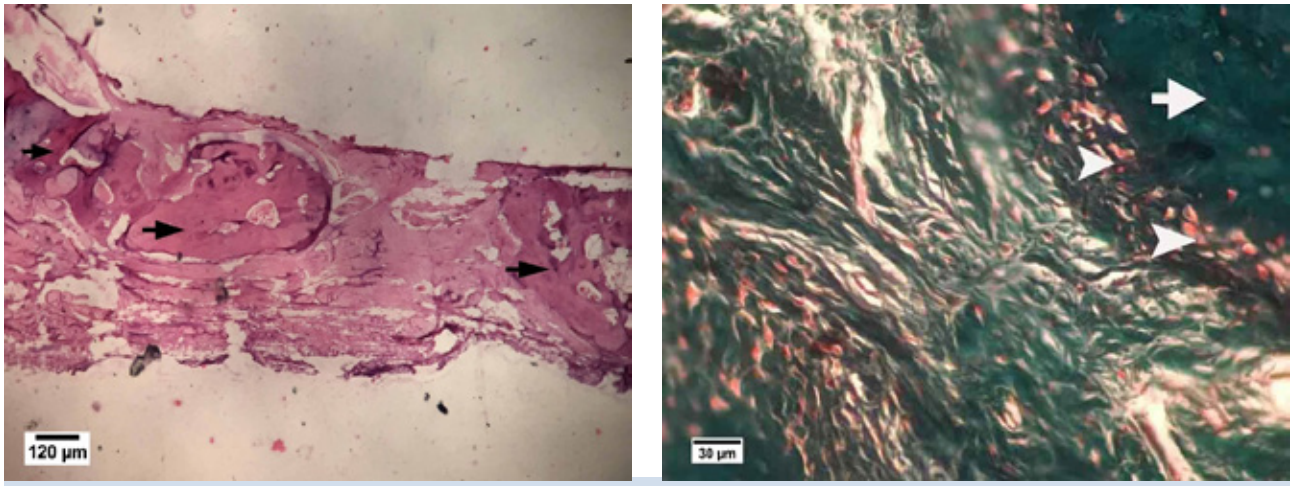


**Figure 12.** Nano group (60-day) that the defect filled with a large amount of immature bone tissue (arrow). Nano group (60-day) that the defect filled with a large amount of immature bone tissue (arrow) (left: H&E, 40×, Right: Masson trichrome, 400×)

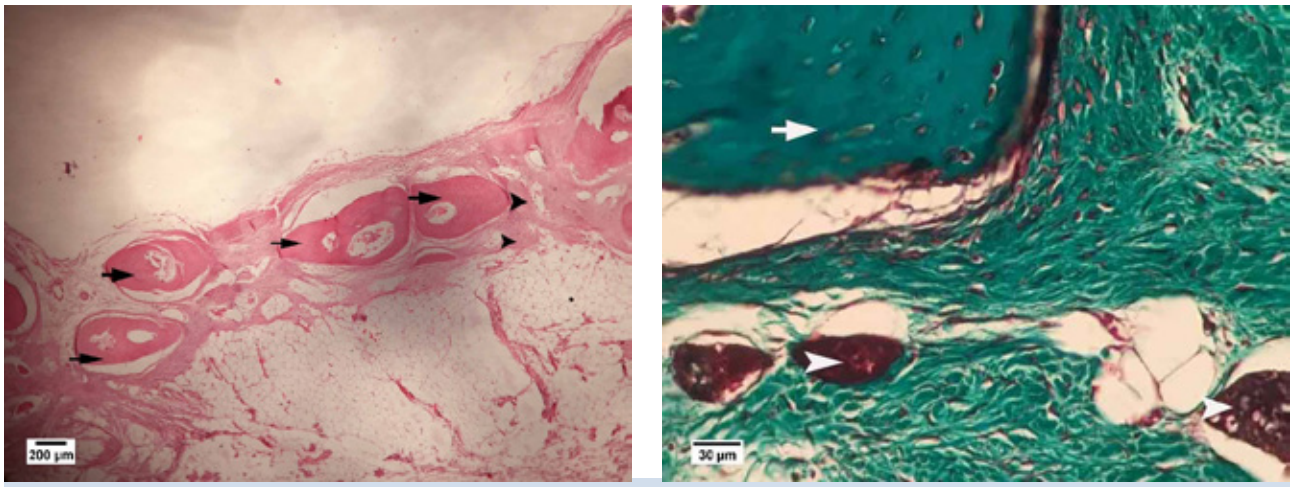


**Figure 13.** Nano autograft group (60-day) that the defect filled with a large amount of immature bone tissue (arrow head) and few cartilage tissues (arrow). Nano autograft group (60-day) that the defect filled with a large amount of immature bone tissue (arrow head) and little cartilage tissue (arrow) (left: H&E, 40×, Right: Masson trichrome, 400×)

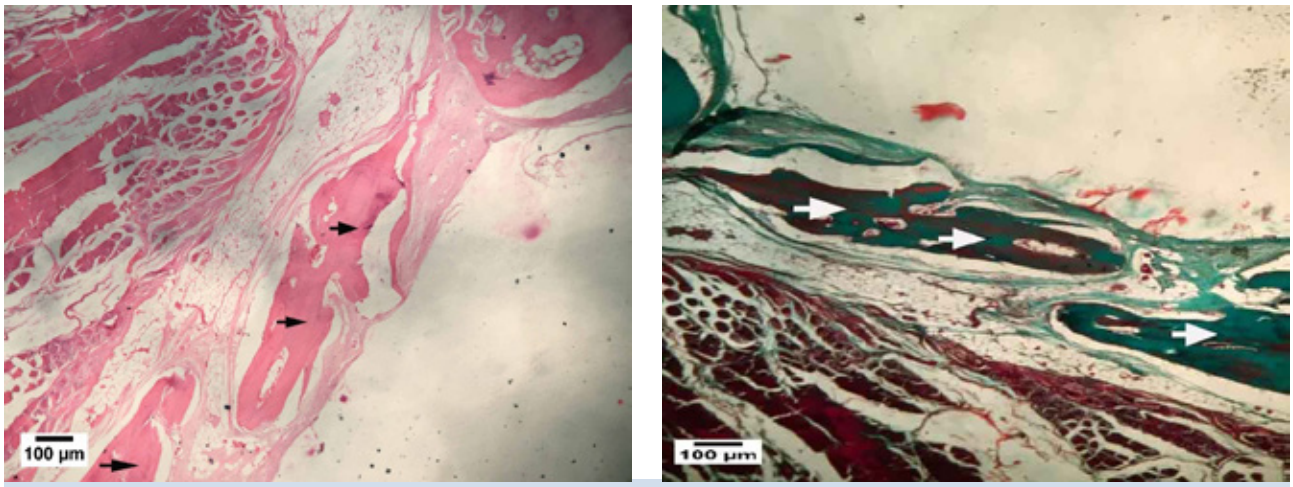


**Figure 14.** Control group (90-day) that the defect filled with a large amount of immature bone tissue (arrow). Control group (90-day) that the defect filled with a large amount of immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 40×)

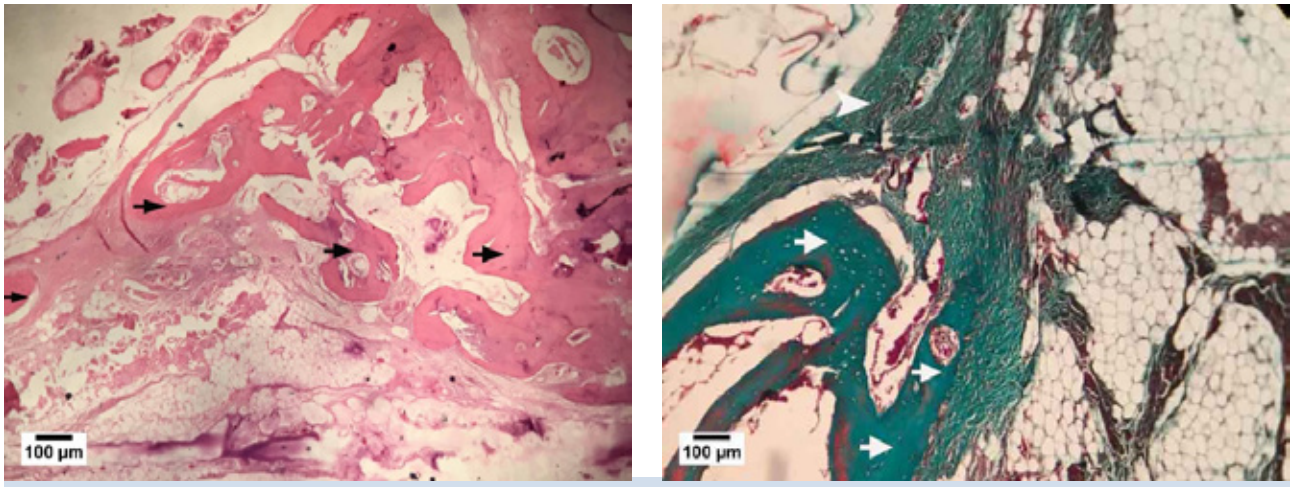


**Figure 15.** Autograft group (90-day) that the defect filled with a large amount of immature bone tissue (arrow). Autograft group (90-day) that the defect filled with a large amount of immature bone tissue (arrow) and few cartilage tissues (arrow head) (left: H&E, 40×, Right: Masson trichrome, 100×)

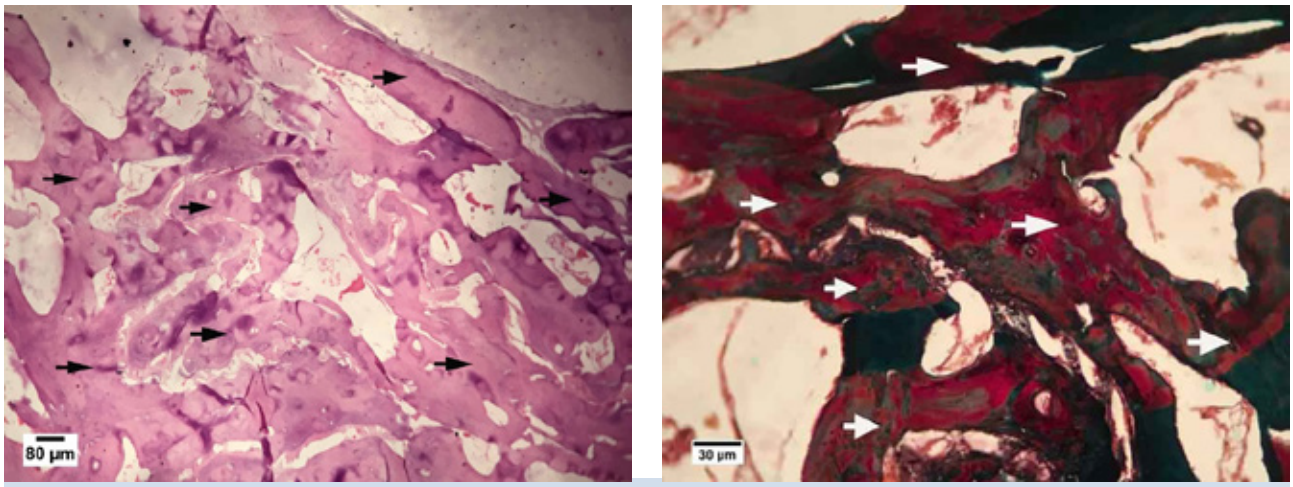


**Figure 16.** Nano group (90-day) that the defect filled with only large amount of immature bone tissue (arrow).Nano group (90-day) that the defect filled with large amount of immature bone tissue (arrow) (left: H&E, Right: Masson trichrome, 400×)

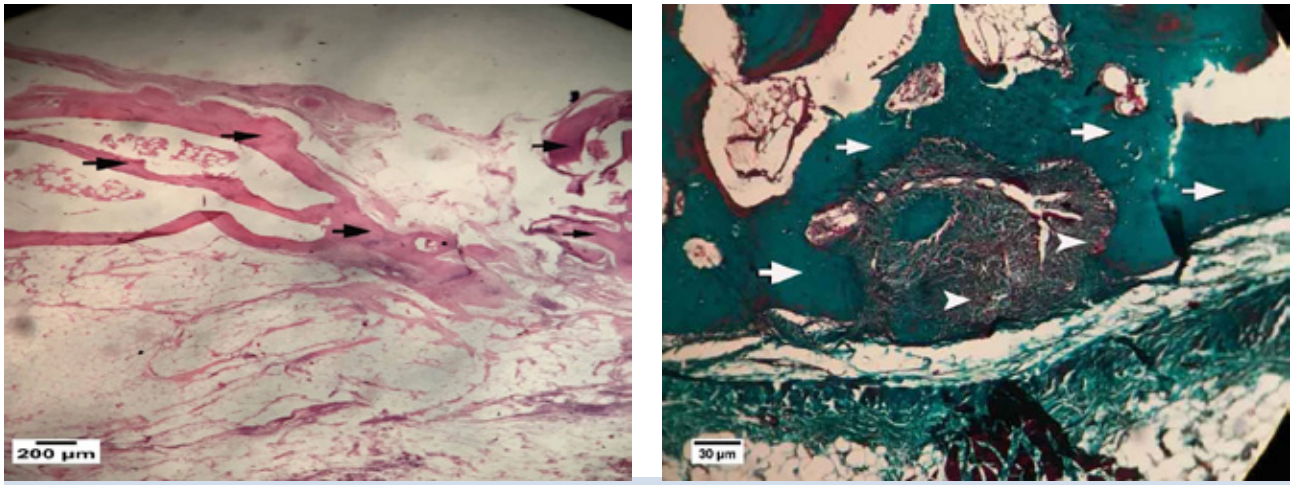


**Figure 17.** Nano-autograft group (90-day) that the defect filled with large amount of immature bone tissue (arrow). Nano-autograft group (90-day) that the defect filled with large amount of immature bone tissue (arrow) and little cartilage tissue (arrow head) (left: H&E, 40×, Right: Masson trichrome, 400×)

**Table 1.** Criteria for scoring histological sections

| Score | Parameter | Criteria |
|---|---|---|
| | Newly formed vessels | None |
| **0** | Numbers of fibroblasts | None to very minimal |
| | Osteoid (bone matrix) | None |
| | Bone | None |
| | Newly formed vessels | Few blood vessels |
| **1** | Numbers of fibroblasts | Few fibroblasts |
| | Osteoid (bone matrix) | Evidence of matrix osteoid |
| | Bone | Evidence of bone formation |
| | Newly formed vessels | Moderate blood vessels number |
| **2** | Numbers of fibroblasts | Predominantly fibroblasts |
| | Osteoid (bone matrix) | Moderate bone matrix deposited |
| | Bone | Moderate bone cells |
| | Newly formed vessels | Extensive blood vessels |
| **3** | Numbers of fibroblasts | Fewer number of fibroblasts |
| | Osteoid (bone matrix) | Dense highly organized bone matrix |
| | Bone | Extensive bone cells |

## DISCUSSION

There is growing interest on producing new biocompatible materials from animal products in regeneration (Kattimani et al., 2014). Avian eggshell has similar physical characteristics to the GBR for space filling (Krishna et al., 2007). Additionally, ostrich eggshell has better effect in regeneration physical characteristics (Kattimani et al., 2014). To determine accuracy of the new biocompatible materials for bone healing, bilateral calvarial defect is the common method because of easy handling and low morbidity by minimize damage to the superior sagittal sinus (Park et al., 2008). In the defects we used bilateral calvarial defect to determine effect of the NCOE on calvaria healing in rabbit. According to the results, filling defect significantly increased in NCOE-treated rabbits compared to the control group at 30- and 60-days post-surgery ($P<0.05$). There was significant difference between experimental groups compared to the control group at 30- and 60-days post-surgery while no differences observed among autograft, NCOE, autograft + NCOE ($P>0.05$). Also, absorb material significantly decreased in NCOE and autograft + NCOE groups compared to the to the control group at 60 days post-surgery. The filling defect significantly increased in autograft, NCOE and NCOE + autograft groups compared to the control group at 90 days post-surgery ($P<0.05$). There was no significant difference on inflammation and material absorption among the groups at 90 days post-surgery ($P>0.05$). The biocompatibility of the filling defect is based on reaction of the surrounding tissue (Yadao et al., 2004). The biocompatibility of the eggshell was expected because the calcium carbonate, as main component of the bone (Yadao et al., 2004). Dense alloplastic materials have higher incidence of extrusion compared to bone grafts (Zingg et al., 1991). The lack of porosity in eggshell implant inhibits the invasion of fibro-vascular network that could help anchor the implant to the underlying bone (Zingg et al., 1991). No change was observed in bone resorption and eggshell graft placed in the craniofacial region of the rabbits 5-20 weeks after implantation (Yadao et al., 2004).

On evaluation of bone healing with eggshell-derived bone graft substitutes in rat calvaria, Park et al. (2008), reported eggshell-treated animal had greater new bone formation and mineralized bone-to-graft contact of surface-modified. Biodegradability and microporous surface structure of bone has key role in bone healing (Park et al., 2008). Biphasic calcium phosphate ceramics has higher osteoconduction than stable hydroxyapatite because of its biodegradability in body fluids and surface microstructures (Furlaneto et al., 2007). Microporosity affects the dissolution rate of bone substitutes in biological fluids (Daculsi et al., 2003). Surface microstructure improves adsorption of proteins and fibronectin which affects cell adhesion, cell proliferation and differentiation. So, based in the above, nano-composite form of the ostrich eggshell was used. Hydrothermal phosphate solutions achieved partial conversion of the particulated hen eggshell to calcium-deficient hydroxyapatite (Rouahi et al., 2006). Calcium-deficient hydroxyapatite has higher biodegradability and rapid surface apatite layer formation and bone bonding (Barrere et al., 2003). These surface properties might contribute to the increased osteoconduction of hydrothermally treated eggshell in the healing of rat calvarial defects (Barrere et al., 2003). The formulations of eggshell used as mineral and trace element supplying agent (Kattimani et al., 2014). In role of the eggshell-derived bone graft substitutes on bone healing in rats, it is reported eggshell-derived bone graft enhances the new bone formation (Uraz et al., 2013).

Small degradation time, poor mechanical possessions and low integrated biological components lead in inability to form, maintain and actively support tissue remodeling. Ostrich shell membranes has gradual degradation than collagen membranes (Park et al., 2008). The main factor for an osteoconductive material is the particle size and foreign body reaction. Based on findings of the current study, no foreign body reaction was observed using NCOE. Based on radiologic report, 50 and 75 μm chick eggshell particles fully absorbed after 60 days and 150-300 μm particles resorbed after 4 months (Dupoirieux et al., 2001). Soluble eggshell matrix proteins are critical for calcium transport (Yadegari et al., 2015). Bone formation and remodelling are controlled by non-collagenous proteins of the bone matrix. Non-collagenous proteins of the bone matrix regulate formation and remodeling (Yadegari et al., 2015). During the chicken eggshell formation, these matrix proteins have effect on calcite crystal morphology. Transforming growth factor β1, lectin-like proteins and Calbindin (calcium binding protein) is isolated form eggshell and stimulate bone formation (Yadegari et al., 2015). Durmus¸ et al., (2003), reported application of the eggshell powder, the outer and inner ostrich eggshell membranes produced little adjunctive effect. Ococalyxin and ovocleidin are eggshell specific proteins and has the eggshell formation in hen's uterus during the chick-

en embryonic development (Neunzehn et al., 2015). Eggshell is ideal source for hydroxyapatite and calcium carbonate (Neunzehn et al., 2015) which promotes the vascularization and wound procedure in the defect edges (Abdulrahman et al., 2014). Eggshell-derived graft substitutes enhance the new bone formation and higher levels of osteoid formation in the eggshell grafted defects (Uraz et al., 2013). It is suggested enhanced bone regeneration in the defect margins (Baliga et al., 1998) which our result was similar to their report. No inflammation, encapsulation and foreign body reaction reported 3 months after treatment, with larger eggshell particles in dog tibial (Yadegari et al., 2015) which our finding was similar to this report. Using standardized defects (8mm diameters) in the parietal bones of rabbit calvaria allowed large increases in their interface with bone graft materials without any effect on the other defects (Takauti et al., 2014).

In conclusion, bone defects occur because of the medical operations, social and economic problems in human (Durmuş et al., 2003). These results suggested NCOE + autograft has positive effects on calvaria healing in rabbit. It seems NCOE has potential efficacy of osteoconductive bone substitute in a rat calvarial defect model. Bone regeneration includes several intra cellular and extra cellular signaling pathways which lead to osteoinduction and osteoconduction to increase bone regeneration in human. It seems, merit researches needed to determine direct cellular and molecular mechanisms of action for further application of the NCOE in clinical trials.

## ETHICAL ISSUES

All protocol of the study was approved by ethic committee of Islamic Azad University, Science and Research Branch, Tehran, Iran.

## CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Occurrence of *Campylobacter*, *Salmonella*, and *Arcobacter* in pet birds of northern Iran

**S. Seifi[1], R. Khoshbakht[1], A. Azizpour[2]**

[1]*Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran*

[2] *Meshginshahr Faculty of Agriculture, University of Mohaghegh Ardabili, Ardabil, Iran*

**ABSTRACT.** Pet birds can harbor human pathogens and contribute to the transmission of infectious agents to human. Since many people are interested in keeping pet birds, this study was conducted in pet birds from Mazandaran province, northern Iran. Totally, 174 fecal samples of pet birds (cockatiel, canary, lovebird, parrot, mynah, goldfinch, budgerigar, macaw, dove, pigeon, and bulbul) were collected with sterile cotton swabs and submitted to Faculty of Veterinary Medicine, Department of Pathobiology (Amol, Iran). After extraction of total DNA, the samples subjected to molecular detection of the Campylobacter, *Salmonella*, and *Arcobacter* using polymerase chain reaction. A total of 114 (65.5%), 28 (16%), and 86 (49.4%) samples were found positive for *Campylobacter*, *Salmonella*, and *Arcobacter*, respectively. Furthermore, some birds showed contamination with two or all three of these bacteria. Results showed that mentioned bacteria can be detected from the apparently healthy pet birds. Therefore pet birds can be considered as potential carriers of these enteropathogens.

***Keywords:*** Pet birds, Enterophatogens, PCR, Fecal samples

*Corresponding Author:*
Saeed Seifi, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, 24th Aftab, Imam Khomeini Street, Amol, Iran
E-mail address: s.seifi@ausmt.ac.ir

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## INTRODUCTION

Infectious diarrhea is a major concern for public health worldwide and it caused by contamination of water and food with pathogenic bacteria, viruses, or parasites. Diarrheal disease is the primary cause of morbidity and mortality among children in developing countries (Kotloff et al., 2013). The common bacteria agents causing diarrhea are *E.coli*, *Salmonella*, *Campylobacter* and with less importance *Arcobacter* (Neupane et al., 2017). Pet birds have an important role as potential vectors of disease, especially regarding human health. People especially children and the elderly interests to keep pet birds, thus there is potential ability to transmit mentioned pathogens to humans.

In recent years, an increased number of human infections with bacteria of the *Campylobacter* type are observed (Grzybowska-Chlebowczyk et al., 2013). Worldwide, campylobacteriosis is the most commonly reported enteric bacterial infection in the human population in developed countries (Lyngstad et al., 2008). Although poultry products are important transmission vehicles to humans, the bacterium is common in pet birds, which live in close contact with humans (Griekspoor et al., 2013). Birds are ideal hosts for campylobacters, due to their relatively high body temperature (42 °C), also, the occurrence of *Campylobacter* spp. in the gut of apparently healthy birds has frequently been reported (Lillehaug et al., 2005). Transmission to humans can occur by aerosols, direct or indirect contact (Belén et al., 2010).

Salmonellosis is an important zoonotic infection seen in all species of animals. A variety of *Salmonella* species have been found in both apparently healthy and obviously diseased birds (Benskin et al., 2009). Transmission to humans was reported in different cases. Salmonellae in humans can cause enteric fever (typhoid) resulting from bacterial invasion of the bloodstream, and acute gastroenteritis resulting from food-borne infection/intoxication (Boseret et al., 2013).

*Arcobacter* spp. have been considered as potential zoonotic foodborne and waterborne agents and can be found in meat (veal, beef, pork and poultry), milk and water (Lehner et al., 2005). Infection in human patients causes diarrhea, abdominal pain and other symptoms including nausea, vomiting and fever. No association of *Arcobacter* with pathologies in poultry has been reported (Vandenberg et al., 2004).

Understanding the spread of bacterial pathogens in pet birds may help as a useful model for examining the spread of other disease organisms, both amongst birds, and from birds to other species. Thus, the aim of this study was to molecular identification of *Campylobacter*, *Salmonella*, and *Arcobacter* from pet or companion birds.

## MATERIALS AND METHODS

**Sampling:** From July to November 2017, 174 fecal swab samples were collected from different species of the companion birds. Fantail pigeon (*Columba livia*) were the most represented species with 19.5% of the samples, followed by Budgerigar (*Melopsittacus undulatus*) with 11.5%, White-eared bulbul (*Pycnonotus leucotis*) 10.3%, Goldfinch (*Carduelis carduelis*) 9.1%, Cockatiel (*Nymphicus hollandicus*) 8%, Common mynah (*Acridotheres tristis*) 6.8%, and Rock pigeon (*Columba livia*) 5.7%. Other bird species composed less than 30% of the samples. All samples placed in separate sterile plastic bags to prevent spills and cross contamination and immediately transported to the laboratory in a cooler with ice packs. All birds were apparently in healthy condition and none received any antimicrobial treatment during the study period.

**DNA extraction and PCR:** The cotton swabs were placed in 1.5-ml tubes in 300 µl peptone water and vortexed thoroughly. Fifty microliters of each fecal suspension was used as input for the DNA extraction procedure. DNA extraction was done using stool DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer recommendations with some modifications. Briefly, 100 mg of each pooled sample was mixed with 20 µl proteinase K and incubated for 10 min at 55 °C. After centrifugation of the mixture at 13000 rpm, the supernatant was mixed with 200 µl binding solution in a new tube and incubated again for 10 min at 60 °C. After incubation, 100 µl isopropanol was added to the tube and then the liquid transferred into the binding column, and centrifuged for 1 min at 8000 rpm. This step was repeated using 500 µl for both washing buffer 1 and 2; then, DNA was precipitated using 100 µl elution buffer and centrifugation at 13000 rpm for 1 min. Extracted DNA was kept at -20 °C until use in PCR. Conventional PCR reaction was done for detection of *Salmonella* spp., *Arcobacter* spp., and *Campylobacter* genus using specific primers (Table 1).

**Table 1.** The primers used in this study for detection of *Salmonella*, *Campylobacter* and *Arcobacter*

| Primer sequence (5' to 3') | Target gene | Annealing temperature (ºC) | Product size (bp) |
|---|---|---|---|
| F:ATCTAATGGCTTAACCATTAAAC<br>R: GGACGGTAACTAGTTTAGTAT | 16S rRNA<br>(*Campylobacter* spp.) | 59 | 875 |
| F: GTGAAATTATCGCCGCCACGTTCGAA<br>R: TCATCGCACCGTCAAAGGAACC | *Inv A*<br>(*Salmonella* spp.) | 58 | 284 |
| F: AGAACGGGTTATAGCTTGCTAT<br>R: GATAVAATACAGGCTAATCTCT | 16S rRNA<br>(*Arcobacter* spp.) | 44 | 181 |

The PCR reaction mixtures consisted of 100 ng DNA template, 2.5 µl 10x PCR buffer (75 mM Tris HCl, pH 9.0, 2 mM MgCl2, 50 mM KCl, 20 mM (NH4)2SO4; Bioneer, Daejeon, So uth Korea), 0.2 mM dNTPs (Bioneer, Daejeon, South Korea), 1.5 U AmpliTaq DNA polymerase (Bioneer, Daejeon, South Korea), and 10 pmol each primer (Takapouzist, Tehran, Iran). The volume of the reaction mixture was reached to 25 µl using distilled deionized water. The thermal cycler (MJ mini, BioRad, USA) was adjusted under optimum conditions. Briefly, Initial denaturation at 94 °C for 4 min, followed by 33 cycles of denaturation at 94 °C for 1 min, annealing as shown in Table 1 for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 7 min. Amplified products were separated by electrophoresis in 1.5% agarose gel electrophoresis stained with ethidium bromide (Cinnaclone, Tehran, Iran). The 100 bp DNA ladder was used as molecular size marker.

**Statistical analysis:** Using Chi-squared, all statistical analyses were performed by SPSS Inc., Chicago, IL (v. 18.0). P value less than 0.05 was considered for statistical significance.

## RESULTS

Among 174 fecal samples collected from pet birds, a total of 114 (65.5%), 28 (16%), and 86 (49.4%) samples were found positive for *Campylobacter*, *Salmonella*, and *Arcobacter*, respectively. Also, simultaneous contamination with *Campylobacter* and *Salmonella* were shown in 12 (6%) of samples. 50 (28%) and 2 (1%) of samples were positive with dual infection of *Campylobacter* and *Arcobacter*, and *Salmonella* and *Arcobacter*, respectively. In 14 (8%) of samples, the presence of all three bacteria were confirmed. Results are summarized in Table 2 and Figures 1 and 2.

**Table 2.** Overall percentages of different types of bacteria isolated from fecal samples collected from pet birds

| Birds (Scientific name) | Name of Bacteria | | |
|---|---|---|---|
| | Campylobacter | Salmonella | Arcobacter |
| Linnet (*Carduelis cannabina*) | 2/4 (50%) | 0/4 (0%) | 4/4 (100%) |
| Goldfinch (*Carduelis carduelis*) | 12/16 (75%) | 0/16 (0%) | 12/16 (75%) |
| Rosy-faced lovebird (*Agapornis roseicollis*) | 8/8 (100%) | 0/8 (0%) | 4/8 (50%) |
| Rose-ringed parakeet (*Psittacula krameri*) | 4/6 (66.6%) | 0/6 (0%) | 4/6 (66.6%) |
| Canary (*Serinus canaria*) | 4/6 (66.6%) | 2/6 (33.3%) | 6/6 (100%) |
| Cockatiel (*Nymphicus hollandicus*) | 10/14 (71.4%) | 2/14 (14.2%) | 6/14 (42.8%) |
| Common mynah (*Acridotheres tristis*) | 2/12 (16.6%) | 2/12 (16.6%) | 4/12 (42.8%) |
| Budgerigar (*Melopsittacus undulatus*) | 8/20 (40%) | 2/20 (10%) | 8/20 (40%) |
| Grey parrot (*Psittacus erithacus*) | 4/8 (50%) | 2/8 (25%) | 2/8 (25%) |
| Alexandrine parakeet (*Psittacula eupatria*) | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |
| Blue-winged parrot (*Neophema chrysostoma*) | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |
| Eastern rosella (*Platycercus eximius*) | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |
| Sun conure (*Aratinga solstitialis*) | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |
| Blue-and-yellow macaw (*Ara ararauna*) | 2/2 (100%) | 0/2 (0%) | 0/2 (0%) |
| Diamond dove (*Geopelia cuneata*) | 2/2 (100%) | 0/2 (0%) | 2/2 (100%) |
| Rock pigeon (*Columba livia*) | 8/10 (80%) | 4/10 (40%) | 0/10 (0%) |
| Old dutch capuchine (*Columba livia*) | 2/6 (33.3%) | 0/6 (0%) | 4/6 (66.6%) |
| Fantail pigeon (*Columba livia*) | 26/34 (76.4%) | 14/34 (41.1%) | 18/34 (52.9%) |
| White-eared bulbul (*Pycnonotus leucotis*) | 12/18 (66.6%) | 0/18 (0%) | 12/18 (66.6%) |
| **Total** | **114/174 (65.5%)** | **28/174 (16%)** | **86/174 (49.4%)** |

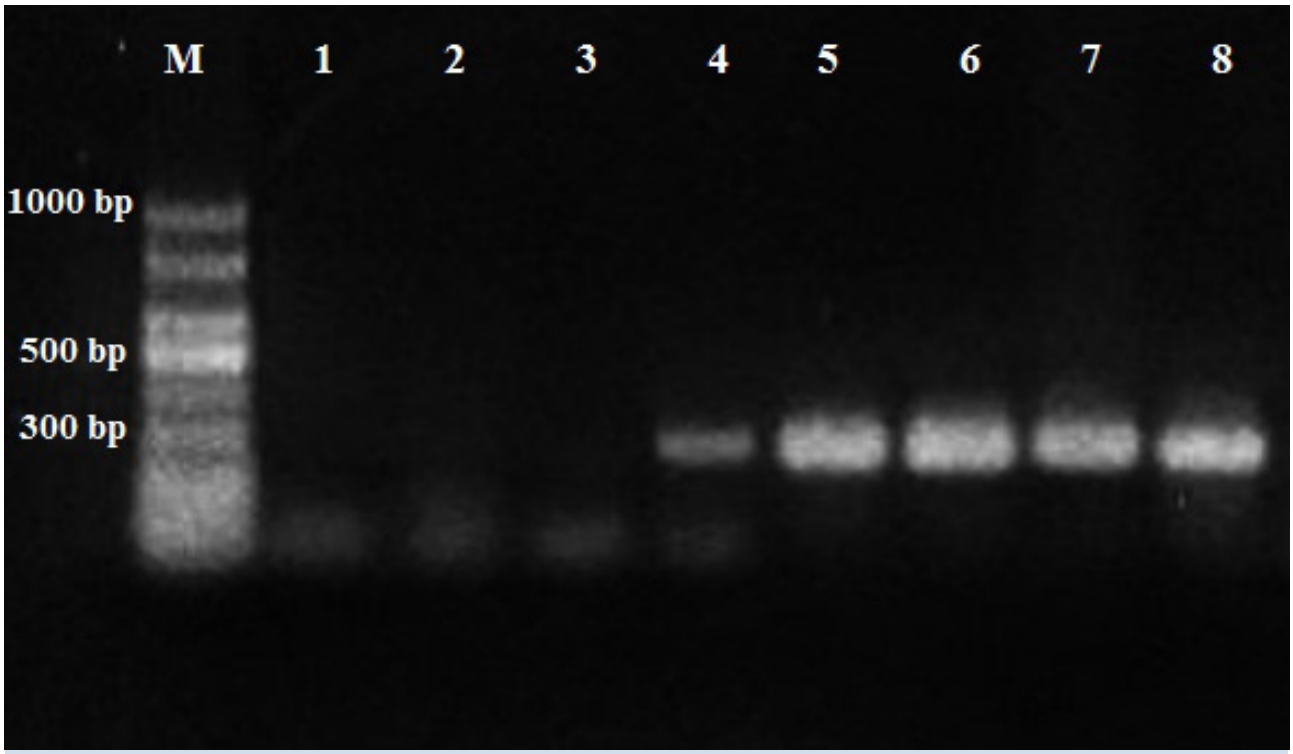


**Fig. 1.** Agarose gel electrophoresis of PCR of the *invA* gene (284 bp) for the characterization of the *Salmonella* species. M: 100-bp ladder as molecular DNA marker; Lane 3: negative control; Lane 4: positive control; Lanes 5-8: positive samples.

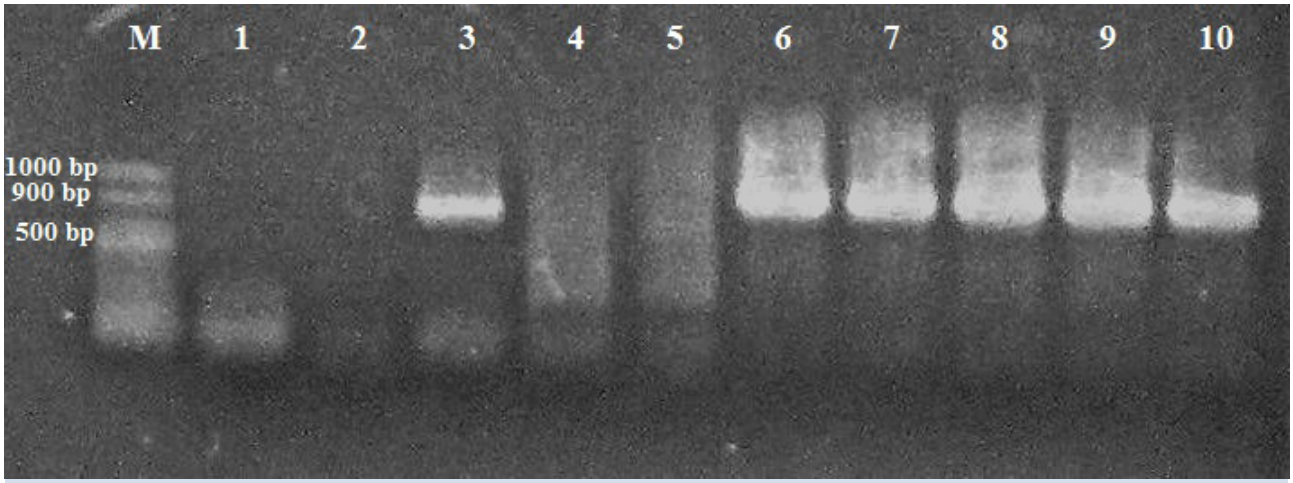


**Fig. 2.** Agarose gel electrophoresis of PCR amplification products using 16S rRNA (857 bp) as a specific primer to identify the *Campylobacter* species. M: 100-bp ladder as molecular DNA marker; Lane 2: negative control; Lane 3: positive control; Lanes 4-5: negative samples; Lanes 6-10: positive samples.

## DISCUSSION

The risk of getting a disease from pet bird is typically highest in people who already have chronic diseases, such as the very young, the elderly, HIV-infected individuals, organ-transplant recipients, and people receiving chemotherapy. Some of the most commonly reported bacterial diseases that can be transmitted through pet birds include Salmonellosis, and Campylobacteriosis. Thus, diagnosis and control of mentioned disease in birds is very important.

Birds are usually considered to be the reservoir of *Campylobacter*, because the body temperature of the birds provides conditions for bacterial growth (Dhama et al., 2013). The overall prevalence of *Campylobacter* spp. for all pet birds sampled in this study was 65.5%. However, the prevalences of *Campylobacter* spp. in members of different pet bird families were

from 16.6% to 100%. Compared with the results of other studies describing *Campylobacter* in pet bird populations, which reported prevalences ranging from 0% to 50% (Waldenstrom et al., 2002; Colles et al., 2008; Zamani Moghaddam et al., 2011; Ehsannejad et al., 2015), the prevalence reported in this study is relatively high. Prevalence differences between studies may due to the use of different sampling and culture methods, which vary in sensitivity. High prevalence rate of *Campylobacter* Spp. (75%) in broiler flocks of Iran was reported by Ansari-Lari et al. (2011), but low detection rate of these bacteria from pet birds mentioned in a previous study (Ehsannejad et al., 2015). In a recent research, Dipineto et al. (2017) reported 13.6% of the cage samples of pet birds were positive for *Campylobacter coli*. One of the possible reasons for the difference in the prevalence can be due to the difference between the bird species. Survival of *Campylobacter* spp. in fecal samples from different bird species is variable (Waldenstrom et al., 2007). Moreover, the type of sample (e.g., cloacal swab vs. fecal sample) and time of sampling (e.g., seasonal variation) collected from birds to assess the prevalence of this organism can influence research findings. The sensitivity of culture techniques should also be considered as a source of variation for prevalence estimates, especially given the fastidious nature of organisms such as *Campylobacter* (Mi'kanatha et al., 2012).

Out of 174 sample tested, 28 samples (16%) were positive for *salmonella* spp. *Salmonella* were detected from Canary, Cockatiel, Common mynah, Budgerigar, Grey parrot, Rock pigeon and Fantail pigeon. Results of present study showed high prevalence of *Salmonella* spp. in pet birds in comparison of other researches. Rahmani et al. (2011), reported from 668 samples tested from birds kept in parks and pet shops in Tehran, Iran, 19 isolates (2.8%) were identified. Similar to some previous study, high prevalence of *Salmonella* spp., was shown in canaries (Georgiades and Iordanidis, 2002; Madadgar et al., 2009; Rahmani et al., 2011). On the other hand, our results is in line with results of Brobey et al. (2017), which reported 17% infection rates of *Salmonella* in wild birds from southeast Texas. In current research, high prevalence of *Salmonella* was detected in pigeons. These results are in agreement to Osman et al. (2013) who reported 33.3% incidence in pigeons. Lower incidence rates were recorded by other researchers who recorded incidence rates of 0%, 3.9%, 4% and 7.9%, respectively (Lillehaug et al., 2005; Tanaka et al., 2005; González-Acuña et al., 2007; Sousa et al., 2010).

High percentage of *Campylobacter* and *Salmonella* detection in this study may be due to geographical and environmental connection. Mazandaran is in the north of Iran (53°6′E, 36°23′N) and located along the southern coast of the Caspian Sea which shares borders with Russia, Kazakhstan, Azerbaijan, and Turkmenistan. Each year when the cold seasons arrive, migratory birds come to Mazandaran wetlands and stay until early March. It is possibility that migratory birds to be an important sources for mentioned bacteria and moving of migratory bird can help to spread infection by direct or indirect contact with carrier, reservoir, Salmonella-infected animals and birds.

Regarding to detection of *Arcobacter* in cloacal swabs, in our study, the highest detection was in Linnet, Canary, Diamond dove, and Goldfinch with incidence rates of 100% for the first three and 75% for the last. Data about the presence of *Arcobacter* spp. in wild birds are rare. This is the first report of *Arcobacter* detection in pet birds in Iran. Di Francesco et al. (2014) showed that 19% detection of *Arcobacter* from Eurasian collared doves in Northern Italy. Khoshbakht et al. (2017) reported no detection of *Arcobacter* from quail farms in Northern Iran. Some researchers showed high isolation of *Arcobacter* from chicken farms (Son et al., 2007; Ho et al., 2008).

In conclusion, this study shows that *Campylobacter*, *Salmonella*, and *Arcobacter* may be excreted in the faeces of apparently healthy pet birds; therefore, pet birds may be a potential source of these bacteria transmission to humans. Furthermore, some birds showed contamination with two or all three of these bacteria. To our knowledge, the molecular detection of *Arcobacter* was reported for the first time in pet birds in Iran. Close physical contact with pet birds that are uncertain about their status, can be a potential risk for public health.

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## CONFLICT OF INTEREST

The authors do not have any potential conflicts of interest to declare.

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Research article
Ερευνητικό άρθρο

# Ultrastructural and molecular characteristics of *Setaria* species based on sequence analysis of genomic and mitochondrial gene markers in cattle (*Bos taurus*) and buffaloes (*Bubalus bubalis*) from Iran

**A. Alborzi[a,*], P. Haddadmolayan[b], M.R.Tabandeh[c], M. Ghorbanpoor[d]**

[a,*] *Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran.*

[b] *Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran.*

[c] *Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran university of Ahvaz, Iran.*

[d] *Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran.*

**ABSTRACT.** The aim of the present study was to investigate the ultrastructural characteristics and genetic diversity of *Setaria* parasites from cattle (n=696) and buffalo (n= 522) from Khuzestan province of Iran and to compare them with available data from other countries/regions by sequences analysis of the 12S rDNA and the mitochondrial cytochrome C oxidase subunit I (*cox1*) genes. Based on SEM (Scanning Electron Micrographs) and light microscopy, all the isolated worms were identified as *Setaria labiatopapillosa*. Our results showed that 12.3% of cattle were infected with *Setaria* spp., while no infection was found in buffaloes. The maximal prevalence was observed in cattle younger than one year old. The prevalence rate was not influenced by the season of the year or gender. Comparison of the obtained sequences from *Setaria* with sequences of *Setaria* spp. from GenBank confirmed that all samples belong to the species *S. labiatopapillosa*. The phylogenetic tree constructed using *cox1* and 12S rDNA genes of several other filarial nematodes showed that the Khuzestan isolates share a common branch with *S. labiatopapillosa* from other regions. Intra-specific variation was observed in 12S rDNA but not in *cox1*. In conclusion, our results indicating that *S. labiatopapillosa* is the main species involved in the spread of setarial infection in south-west of Iran and the identified worms corresponded mostly to worms that reported previously throughout other continents.

***Keywords:*** *Setaria species*; Ultrastructural; phylogenetic; Iran.

*Corresponding Author:*
M. Ghorbanpoor, Golestan Blvd-Ahvaz- Khuzestan- Iran
E-mail address: a.alborzi@scu.ac.ir

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## INTRODUCTION

Filariasis in man and animals is considered as a major health hazard with important medical, veterinary, and economic consequences, affecting millions of people and animals globally (World Health Organization, 2007). *Setaria* species (Family: Onchocercidae and Subfamily: Setariinae) are filarial nematodes that mainly inhabit the peritoneal cavity of ungulates and rodents. *S. digitata, S. marshalli, S. cervi* and *S. labiatopapillosa* are the main species that have been reported from cattle and buffaloes (Anderson, 2000; Islam et al., 1992). Several species of mosquitoes, including *Aedes*, *Culex*, *Armigeres*, *Anopheles* and *Haematobia* fly are vectors for *Setaria* spp. (Anderson 2000; Cancrini et al., 1997). Furthermore, there are some reports about the congenital transmission of *S. digitata* and *S. marshalli* in cattle (Fujii et al., 1995; Wee et al., 1996; Anderson, 2000).

Setariosis is a polyorganic parasitic disease. The migration of adult worms to the bladder, heart, lung, mesenteric lymph nodes, liver, spinal cord and eyes have been reported. Adult worms are not pathogenic in the peritoneal cavity, although they may cause a mild fibrinous peritonitis in definitive hosts (Golovko and Shchetinsky, 2005; Sundar and D'Souza, 2015). Ectopic migration of microfilariae in the central nervous system of nonspecific hosts (sheep, horses, goats and man) and sometimes even in the specific host can cause cerebrospinal nematodiasis or cerebrospinal setariosis that is recognized with clinical signs such as ataxia, lack of motor coordination and paralysis (Tung et al., 2003; Mahmoud et al., 2004). Additionally, in some areas of the world such as Iran and Romania, human infection with larval and adult stages of *Setaria* spp. have also been reported (Talu et al., 2012; Nabie et al., 2017).

Several methods have been used for epidemiological studies and discrimination of species diversity. Traditionally, these worms are identified based on morphological features including cuticular ring, dorsal, ventral and lateral lips, lateral appendages, mouth opening, terminal knob in female worms and spicules and patterns of cloacal papillae in male worms (Shoho and Uni, 1977). Conventionally, the light microscope is used to identify the various species of *Setaria* spp. but, additional studies are needed to evaluate and confirm their morphological variations. SEM (Scanning Electron Micrographs) is a powerful tool with high quality that may facilitate the identification of the *Setaria* spp. morphological variations (Almeida et al., 1991; Ronghang and Roy, 2013; Kumar and Kumar, 2016). So far, no study has been carried out to determine the morphological differences between *Setaria* species in Iran.

Genetic diversity of the parasite spp. may produce different phenotypes which can be associated with host-parasite interaction (Brunner and Eizaguirre, 2016; Viney and Diaz, 2012). Although a few light microscopic studies have been reported on the prevalence of *Setaria* in the cattle and buffaloes particularly from north of Iran (Dawoodi, 2014; Bazargani et al., 2008; Khedri et al., 2014; Nabie et al., 2017), there is no information about the prevalence of setariosis in the southern Iran and ultrastructural and molecular characteristics of parasites have not been studied so far. Thus, the purpose of this study was to determine the prevalence, ultrastructural and phylogenic characteristics of *Setaria* spp. in cattle and buffaloes in Khuzestan province of Iran.

## MATERIALS AND METHODS

### Sampling

During the period from November 2014 to October 2016, a total of 696 cattle (437 male and 259 female) and 522 buffaloes (341 male and 181 female) in different age groups (Table 2) and examined for detection of infection with *Setaria* spp. The slaughterhouse of Ahvaz is a centre for receiving animals from different areas of Khuzestan province. After slaughtering the animals, blood samples were taken from each one, and the abdominal cavity was carefully examined for the presence of *Setaria* spp. The worms were collected and transferred to containers containing phosphate buffered saline (pH = 7.4). Season of sampling, the number of isolated worms, gender and age of each animal were recorded. Some of the specimens were fixed in 70% ethanol for light microscopic examination and DNA extraction. The Modified Knott's technique was used to detect microfilariae in the blood as described by Watanabe et al. (2004), and the numbers and length size of microfilariae were recorded.

### Morphological examination

After the relaxation of the worm samples in hot water and transparency within lacto phenol, they were mounted using the glycerine-gelatine solution and examined under light microscope.

The methods of Ronghang and Roy (2013) and Kumar and Kumar (2016) with some modification were used for ultrastructural study of samples by

SEM. Briefly, worms were washed in PBS, and the anterior and posterior ends of the worms were cut and fixed in 5% glutaraldehyde (Sigma, USA) for 24h at 4 ˚C. Some samples were post fixed in 1% osmium tetroxide (Sigma, USA) for 4h at 4 ˚C. Then the dehydration process of all samples was performed with a gradient series of ethanol at 4 ˚C and the samples were dried using Tetramethylsilane (Sigma, USA). Finally, specimens were mounted on aluminium stubs, coated with a thin layer of gold, and examined with Leo 1455 VP SEM (Carl-Zeiss, Germany) at 18-23 KV. *Setaria* species were determined according to diagnostic keys (Shoho and Uni, 1977).

**PCR Amplification**

Template DNA from whole blood samples and adult *Setaria* worms were extracted using Genomic DNA extraction Kit (SinaClon Bioscience, Iran) according to manufacturer's instruction. In order to identify microfilariae in blood samples that were negative based on modified Knott's test (97 cattle and 30 buffaloes), PCR reaction was performed on tandemly repeated DNA using M2F and M2R primers as described by Wijesundera et al. (1999). For molecular characterization and phylogenetic analysis of *Setaria* spp., mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and 12S rDNA genes were amplified by PCR according to methods that previously described by Yatawara et al. (2007) and Casiraghi et al. (2001). Characteristics of primers that were used in the present study are shown in table 1.

**Table 1.** PCR conditions and primer sequences for tandemly repeated DNA, *cox1* and 12Sr DNA.

| Target gene | Organism | Primer sequence (5′→3′) | Annealing temperature (°C) | Product size (bp) |
|---|---|---|---|---|
| tandemly repeated DNA | Microfilariae | M2F:CCGACATCAAGTTCATG<br>M2R: GATTCAAGAACATGGTG | 48 | Ladder like |
| *cox1* | Adult worm | COX1intF: TGATTGGTGGTTTTGGTAA<br>COX1intR: ATAAGTACGAGTATCAATATC | 54 | 680 |
| 12S rDNA | Adult worm | 12SF:GTTCCAGAATAATCGGCTA<br>12SR:ATT GACGGATG (AG) TTTGTACC | 54 | 450 |

All PCR reactions were performed in a 20 µl volume containing: 10 µl of Taq DNA polymerase master mix Red (Amplicon, Denmark, MgCl2: 1.5 mM), 0.5 µl of each primer (10 µM) (Macrogen, South Korea) and 3 µl of DNA template (~100 ng) and 6 µl of DNase free water. PCR cycling included an initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, specific annealing for 45s, and extension at 72 º C for 1 min. Then a final extension at 72ºC for 5 min was performed as well. PCR reactions included a negative control, consisting of the reaction mix and 3 µl of DNase free water instead of template DNA. A positive control consisting of DNA sample isolated from intact worm was used for PCR reaction on blood samples. PCR products were electrophoresed on 1.5% agarose (SinaClon Bioscience, Iran) in Tris-acetate-EDTA (TAE) buffer, stained with Green SafeStain (SinaClon Bioscience, Iran) and visualized under ultraviolet light.

**Phylogenetic analyses**

PCR products of *cox1* and 12s rDNA were purified using Gel recovery kit (Vivantis, Malaysia) and sequenced on both strands using Big Dye Terminator V.3.1 Cycle Sequencing kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Sequences were aligned together using ClustalW (Larkin et al., 2007) software to determine the consensus sequences. Consensus 12S rDNA and cox1 sequences were subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) and compared to all nucleotide sequences of *Setaria* species available in the current databases. Sequence identities (in %) were calculated by pairwise comparisons. Subsequently, the consensus sequences were aligned with a selected subset of closely related sequences of the genus *Setaria*. Phylogenetic relationships were inferred based on analyses employing the Neighbor-Joining (NJ) method using MEGA7. The topological sta-

bility of the tree was evaluated by 1000 bootstrap replications.

**Statistical analysis**

The findings of this study were analyzed using SPSS software (version 21). The associations between age, gender, season and infection were analyzed by Chi-square test ($X^2$-test). The level of significance was at 5%.

## RESULTS

**Prevalence**

As shown in table 2, 12.3% (95% CI: 9.9-14.8%) of cattle were infected with *S. labiatopapillosa*, while no infection was found in buffaloes. The prevalence rate of infection with adult worm, microfilariae and the both were 9.77% (68 out of 696), 8.76% (61 out of 696) and 6.17% (43 out of 696), respectively. Out of the 437 male cattle screened, 56 (12.8 %) were infected, while the prevalence rate of infection in females was 11.5% (30 out of 259). There was no statistically significant difference in infection between genders (P= 0.633). The prevalence of infection in cattle younger than one year, one to three years and more than three years was 19.18% (40 out of 228), 9.79% (28 out of 286) and 9.89% (18 out of 182), respectively. There were significant differences between age groups and infection (P= 0.015). The prevalence rate of infection in spring, summer, autumn and winter was 16.57%, 12.19%, 9.45% and 11%, respectively. The prevalence of infection with *Setaria* in spring (16.57%) was greater than other seasons, but there was no statistically significant difference between different seasons (P=0.224) (table 3).

**Table 2.** Prevalence of *Setaria* infection in cattle and buffaloes.

| Animals | Gender | | Total | Age | | |
|---|---|---|---|---|---|---|
| | Male | Female | | <1 | 1 - <3 | 3 - >3 |
| Cattle examined | 437 | 259 | 696 | 228 | 286 | 182 |
| Infected Cattle (%) | 56 (12.8) | 30 (11.5) | 86 (12.3) | 40 (17.5) | 28 (9.7) | 18 (9.8) |
| Buffalos examined | 341 | 181 | 522 | 133 | 247 | 142 |
| Infected buffaloes (%) | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3.** Prevalence of *Setaria* infection in cattle based on gender, season and age.

| Variables | Parameters | No. examined | No. positive (%) | *P*-value |
|---|---|---|---|---|
| Gender | Male | 437 | 56 (12.8) | 0.633 |
| | Female | 259 | 30 (11.5) | |
| Season | Spring | 175 | 29 (16.57) | 0.224 |
| | Summer | 164 | 20 (12.19) | |
| | Autumn | 148 | 14 (9.45) | |
| | Winter | 209 | 23 (11) | |
| Age | <1 | 228 | 40 (19.18)[a] | 0.015 |
| | 1 - <3 | 286 | 28 (9.79) | |
| | 3 - >3 | 182 | 18 (9.89) | |
| **Total** | | 696 | 86 (12.3) | |

* Figures in columns with different characters are statistically significant.

The first stage microfilariae were isolated from 61 out of 696 (8.76%) blood samples of cattle by the modified Knott's test. PCR amplification of tandemly repeated DNA of *Setaria* genome in negative blood samples (negative on modified Knott's test) revealed that 47.42% (46 out of 97) of cattle blood samples were positive and ladder-like pattern was seen in gel electrophoresis (Fig. 3A) while no filarial DNA was detected in blood samples of buffaloes. This suggests that Knott's test (8.76%) in comparison to PCR technique (47.42%) cannot indicate up to thirty percent (38.6%) of the negative blood samples.

**Morphology**

A total of 101 worms (4 male and 97 female) were isolated from examined cattle. The average number of recovered worms in each animal was 1.36 (range: 1-15). All isolated worms were identified as *S. labiatopapillosa* based on light microscopy and SEM. The average lengths of male and female worms were 4.9 and 9.6 cm, respectively. At the anterior end of female and male worms, cuticular peribuccal ring existed with dorsal and ventral lips with notched elevation, lateral lips and elliptical shaped mouth opening (Fig. 1A, 1B and 2A). A pair of amphids with cuticular mosaic appearance was located in the lateral sides of the peribuccal ring (Fig 1C and 1D). Genital pore in female worms was situated on the ventro-lateral side (Fig 1A and 1B). Lateral appendages were seen at posterior end in both sexes (Fig 1E and 1G). A knob was detected at the end of the tail in female worms with blunt papilla and transverse lines at the base (Fig. 1E and 1F). Male *S. labiatopapillosa* had coiled tail with three pair of pre-cloacal, one pair ad-cloacal and four pair of post-cloacal papilla. Furthermore, ventral bands and a single blade-like spicule emerging from the cloaca were seen at the posterior end of male worms (Fig. 1G and 1H).

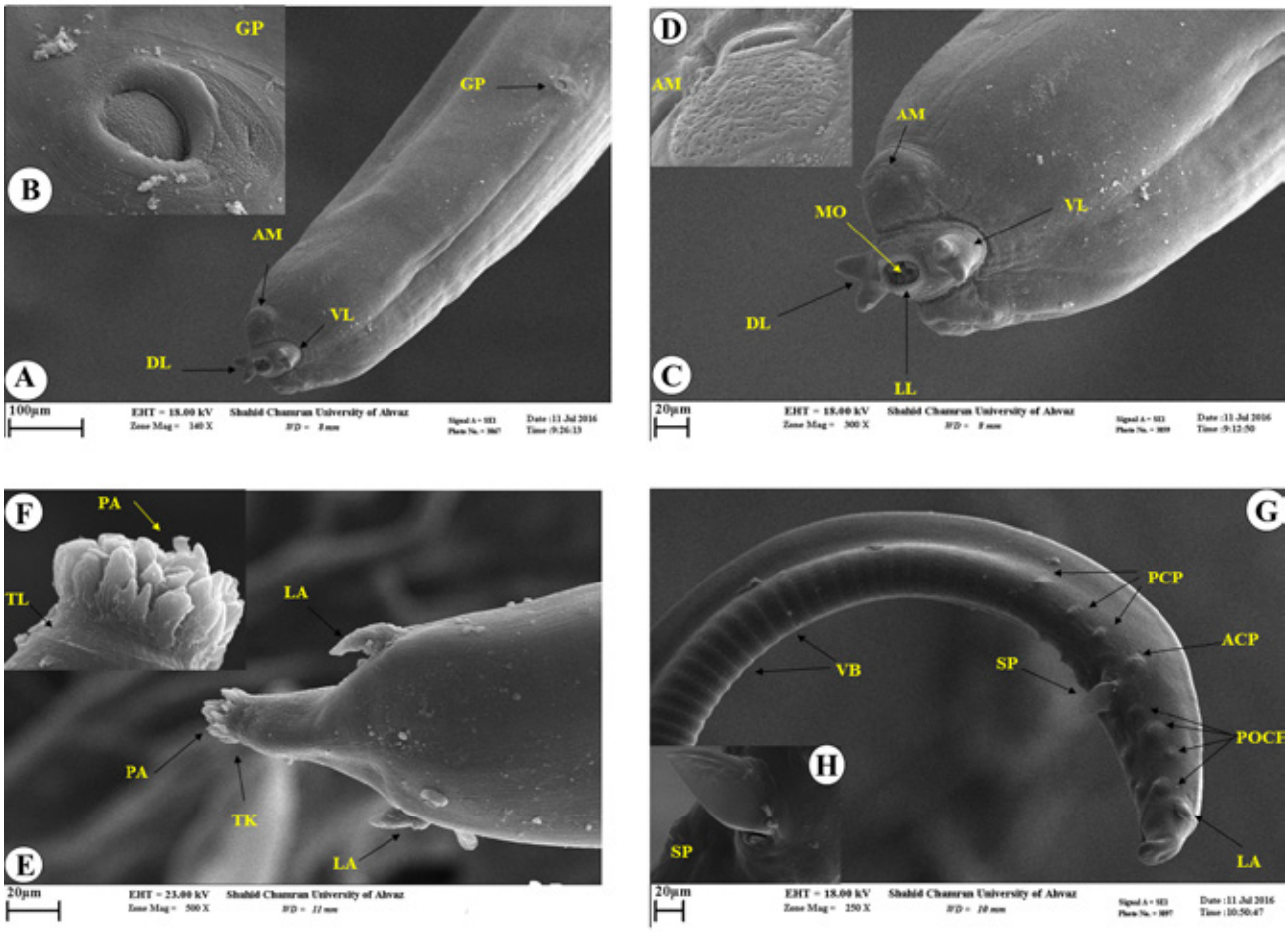



**Fig. 1.** Morphological characteristics of *S. labiatopaoillosa* studied by SEM. A: Anterior end of Female worm (140X). B: Genital pore (3000X). C: Anterior end of Female worm (300X). D: Amphid (3000 X). E: Posterior end of Female worm (500X). F: Blunt papilla and transverse lines at the tail end in female worm (3000X). G: Posterior end of male worm (250X). H: Spicule (3000X). ACP: Ad-Cloacal Papilla, AM: Amphid, DL: Dorsal Lip, GP: Genital pore, LA: Lateral Appendages, LL: Lateral Lips, MO: Mouth Opening, PA: Papilla, PCP: Pre Cloacal Papilla, POCP: Post-Cloacal Papilla, SP: Spicule, TK: Terminal Knob, TL: Transverse Lines, VB: Ventral Bands and VL: Ventral Lip.

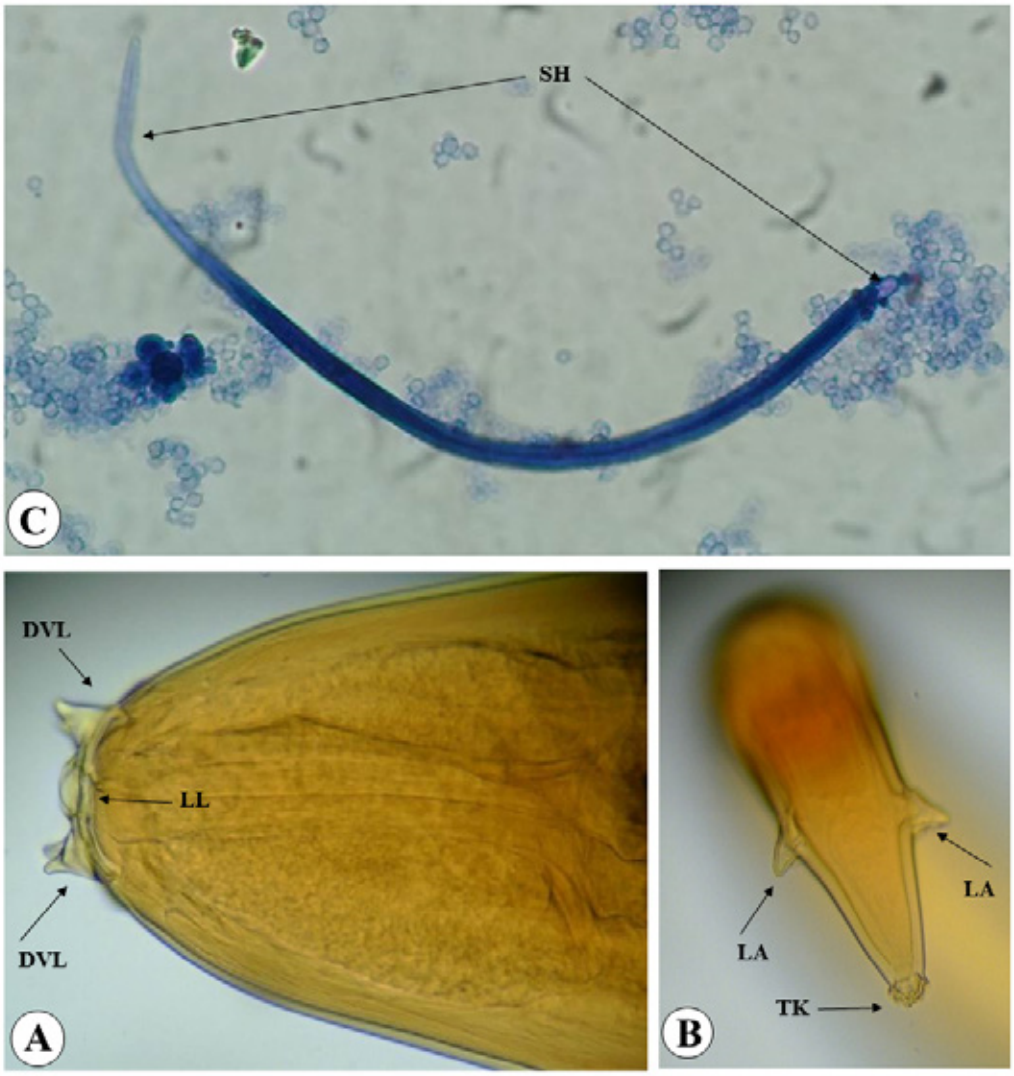



**Fig. 2.** Light Microscopic Images of adult female *S. labiatopaoillosa* and microfilariae. A: Anterior end of Female. B: Posterior end of Female worm. C: Sheathed first stage microfilaria in blood of infected cattle. DVL: Dorsal or Ventral Lips. LA: Lateral Appendages, LL: Lateral Lips, SH: Sheath and TK: Terminal Knob.

The average length of sheathed first stage microfilariae was 304.6 µm (Fig. 2C). The average number of microfilaria in blood samples of infected cattle was 537.25 ±74.89 /ml (range: 45-2520/ml).

**Molecular characteristics of *cox1* and 12S rDNA genes**

PCR amplification of the *cox1* and 12S rDNA genes of *S. labiatopapillosa* produced 680 and 450 base pairs fragments, respectively (Fig. 3B). The DNA sequences of *cox1* (6 variable sites) and 12S rDNA (53 variable sites) obtained in the present study shared 82-99% identities with other *Setaria* species (Table 4 and 5). DNA sequences obtained in the present study showed the highest identity (>98%) to *cox1* and 12S rDNA of *S. labiatopapillosa*, while the lowest similarity was found to sequences from *S. equina*.

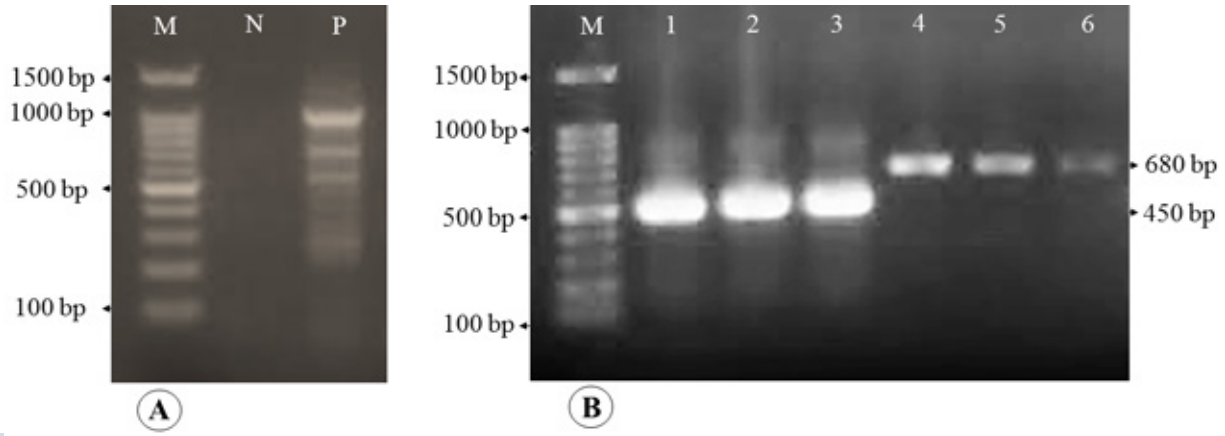



**Fig. 3.** A: PCR amplification of tandemly repeated DNA isolated from blood of cattle infected with *S. labiatopaoillosa* microfilariae (N: negative control, P: positive sample). B: The PCR products of 450 bp and 680 respectively, for *cox1* (1-3) and 12s rDNA (4-6) obtained from adult worm. Lane M is a 100 bp ladder.

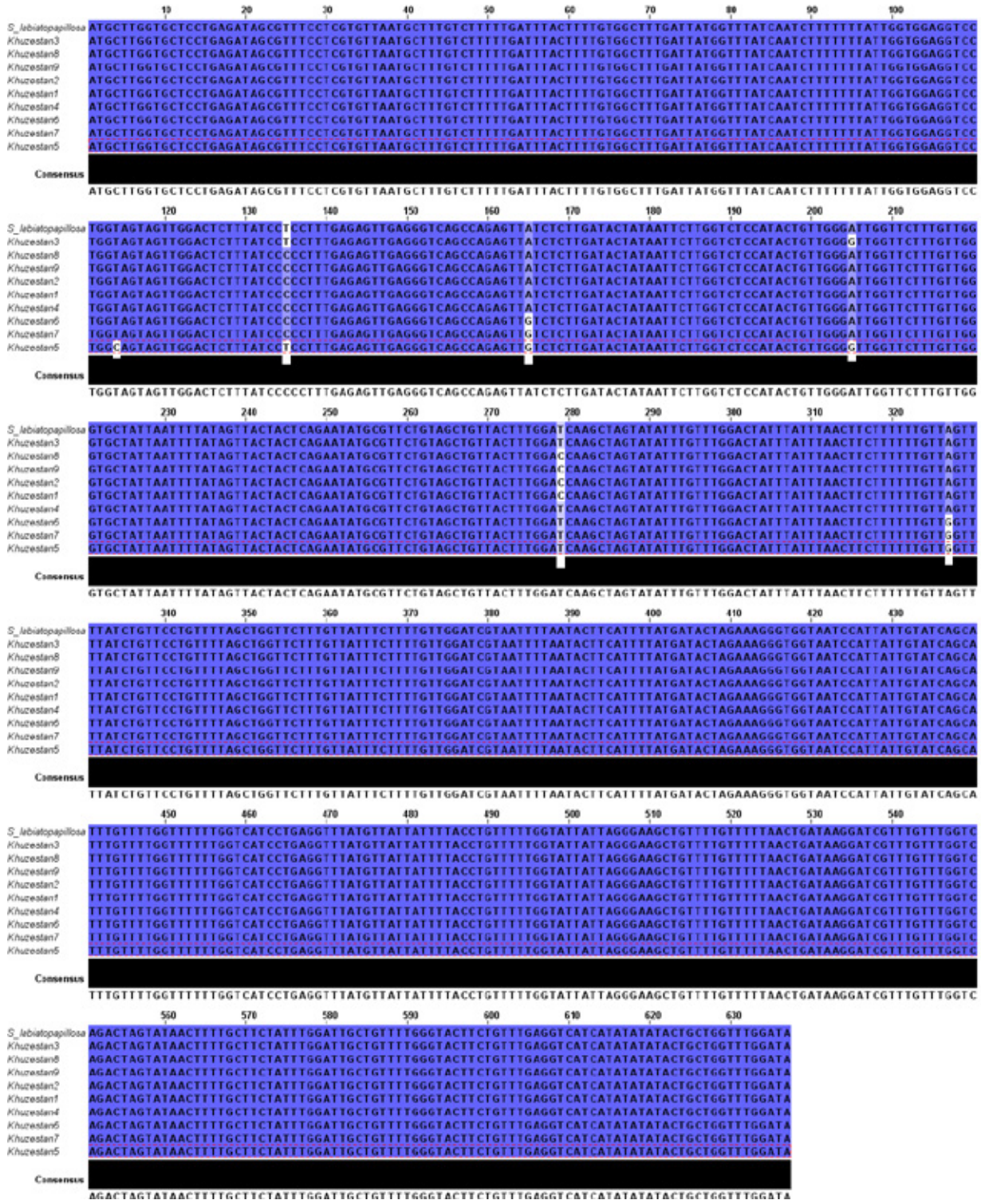
**Table 4.** Similarity values of *cox1* gene of *S. labiatopapillosa* obtained from cattle of Khuzestan province and those from other filarial species and related nematodes. Data was analysed using nBLAST tool.

| Species | Accession No. | Maximal identity (%) |
|---|---|---|
| *Setaria labiatopapillosa* | AJ544872.1 | 99 |
| *Setaria digitata* | EF174425.1 | 91 |
| *Setaria cervi* | JF800924.1 | 91 |
| *Setaria tundra* | KF692106.1 | 91 |
| *Dirofilaria repens* | KX265048.1 | 90 |
| *Setaria equina* | AJ544873.1 | 90 |
| *Wuchereria bancrofti* | JQ316200.1 | 90 |
| *Brugia timori* | AP017686.1 | 89 |
| *Onchocerca ochengi* | KX181289.1 | 89 |
| *Dipetalonema evansi* | KR184805.1 | 88 |
| *Brugia pahangi* | AP017680.1 | 88 |

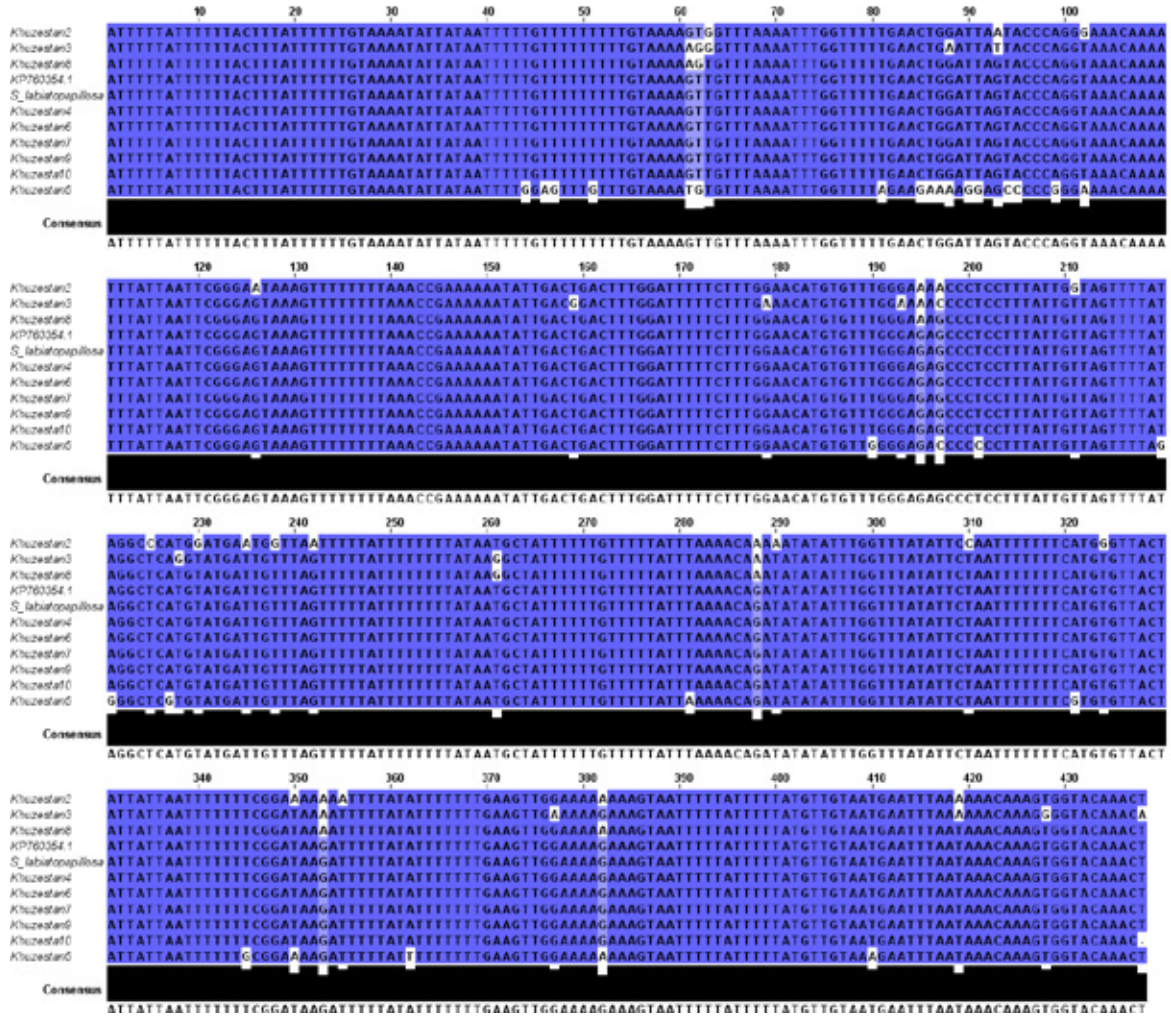
**Table 5.** Similarity values of 12S rDNA gene of *S. labiatopapillosa* obtained from cattle of Khuzestan province and those from other filarial species and related nematodes. Data was analysed using nBLAST tool.

| Species | Accession number | Maximal identity (%) |
|---|---|---|
| *Setaria labiatopapillosa* | KP760354.1 | 98 |
| *Setaria digitata* | EF179382.1 | 89 |
| *Setaria tundra* | AM779828.1 | 87 |
| *Setaria equina* | KU291446.1 | 82 |
| *Piratuba scaffi* | AM779831.1 | 82 |
| *Cercopithifilaria tumidicervicata* | AM779788.1 | 82 |
| *Litomosoides brasiliensis* | KP760336.1 | 81 |
| *Thelazia gulosa* | AJ544857.1 | 80 |
| *Thelazia callipaeda* | LK984781.1 | 77 |

Sequences of *cox1* and 12S rDNA (supplementary files 1 and 2) from all isolates had more than 99% similarity to each other and to *S. labiatopapillosa* sequences that have been previously deposited in GenBank (12S rDNA: KP760354.1, *cox1*: AJ544872.1). 12S rDNA sequences were deposited in GenBank under the accession numbers MF589577, MF589578, MF589579 and MF589580. *Cox1* sequences were deposited in GenBank under the accession numbers MF589581, MF589582, MF589583, MF589584 and MF589585.




**Supplementary 1.** Multiple sequence alignment of the *cox1* sequences of *S. labiatopaoillosa* obtained from cattle of Khuzestan province and Reference GeneBank sequence (Accession NO: AJ544872.1) using ClustalW alignment tool. Different nucleotides in each position are shown with no colour while identical amino acid residues are colored.



**Supplementary 2.** Multiple sequence alignment of the 12S rDNA sequences of *S. labiatopaoillosa* obtained from cattle of Khuzestan province and Reference GeneBank sequence (Accession NO: KP760354.1) using ClustalW alignment tool. Different nucleotides in each position are shown with no colour while identical amino acid residues are colored.

According to the phylogenetic tree of *cox1* (Fig 4), the Khuzestan isolates were clearly grouped with *S. labiatopapillosa* in one node that had high bootstrap values for NJ (0.98). However, sequence analysis showed slight variability within the Khuzestan isolates, which was reflected in their topology in the phylogenetic tree. Accordingly, the Khuzestan isolates were grouped in two clusters. Cluster I (1, 2, 3, 4, 8 and 9 isolates) were more closely related with *S. labiatopapillosa* when compared with cluster II (5, 6, 7 isolates). Due to the lower bootstrap values (0.62), other *Setaria* species including *S. digitata*, *S.cervi*, *S.tundra* and *S. equina* were grouped in separate clades based on *cox1* sequence.

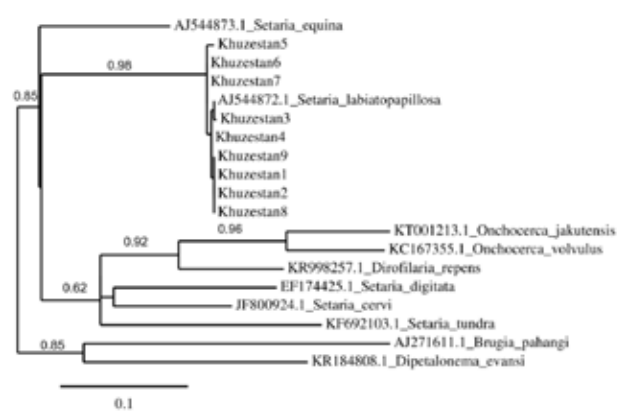



**Fig. 4.** Neighbor-Joining phylogenetic relationship of 9 isolates of *S. labiatopapillosa* from cattle of Ahvaz, Iran. The analysis was based on *cox1* gene sequences (680 bp). Percentage bootstrap support (more than 50%) from 1000 replicate samples is indicated at the right of the supported node. Accession numbers for sequences obtained from GenBank are shown, followed by different Filarioidea species. The scale bar indicates distance.

As shown in Fig 5 based on 12s rDNA sequences of Khuzestan isolates and the other 10 filarial nematodes downloaded from GenBank, all Khuzestan isolates with *S. labiatopapillosa* were grouped in one clade. Other *Setaria* spp. including *S. cervi*, *S. digitata*, *S. tundra* and *S. equina* formed a sister clade well separated from the one consisting of *S. labiatopapillosa* and Khuzestan isolates. Based on 12s rDNA sequence Khuzestan isolates divided into two subclades. Isolates 1, 4, 6, 7, 9 and 10 showed the highest relationship with *S. labiatopapillosa*, while isolates 2, 3 and 8 had the highest genetic variation in relation to *S. labiatopapillosa*.

Our results have also shown that intra-specific variation was observed in *cox1* but not in 12S rDNA.

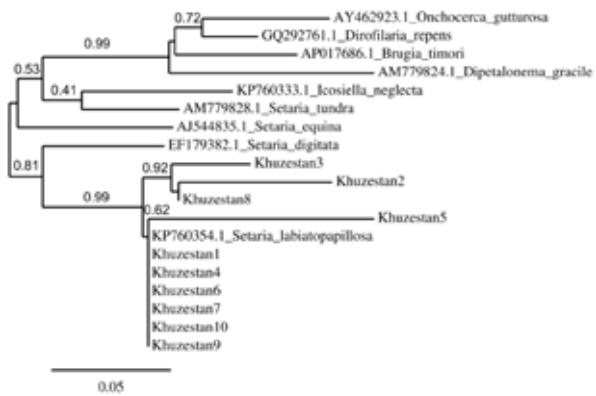



**Fig. 5.** Neighbor-Joining phylogenetic relationship of 10 isolates of *S. labiatopapillosa* from cattle of Ahvaz, Iran. The analysis was based on 12s rDNA gene sequences (450 bp). Percentage bootstrap support from 1000 replicate samples is indicated at the right of the supported node. Accession numbers for sequences obtained from GenBank are shown, followed by name of Filarioidea species. The scale bar indicates distance.

## DISCUSSION

Setariosis is one of the most important parasitic disease in cattle which caused by different species of *Setaria* in different countries of the world. The implementation of molecular techniques for the identification of the parasite species is an important requirement to plan effective control strategies for the emerging parasite infections. This study was undertaken to evaluate the morphological and phylogenetic characteristics of *Setaria* spp. in cattle and buffaloes in Southwest of Iran (Khuzestan region) The climatic conditions of Southwest of Iran favour the presence of mosquitoes at almost 5 months of the year. Sixteen species of mosquitoes belonging to five genera, including *Aedes* (2 Species), *Anopheles* (5 Species), *Culex* (6 Species), *Culiseta* (2 Species) and *Ochlerotatus* (1 Species) were reported from this region which can transmit *Setaria* spp. (Navidpour et al., 2012; Nasirian et al., 2014; Maghsoodi et al., 2015; Farhadinejad et al., 2015).

In this study, the overall prevalence of Setarial infection in cattle was 12.3%, which reflects the considerable prevalence rate of infection in cattle. There were no significant association between seasons and infection which might be due to high longevity of adult worms, absence of periodicity of microfilaria in the blood and seasonal distribution of mosquitoes as well. Our findings showed that the prevalence rate of infection in cattle below 1 year of age was higher than other age groups. This might be attributed to the weakness of the immune system in younger animals and providing more opportunities for the development of the parasite. It seems that the onset of the parasite infection and its complete development occur in calves; thus allowing them to continue in older animals.

Despite the fact that female animals are slaughtered at an older age, no significant differences were observed between the gender and the infection. Several surveys have been carried out to determine the prevalence of *Setaria* spp. around the world. The prevalence ranges from 11.11% to 47% in different regions (North, Northwest and East) of Iran have previously been reported (Bazargani et al., 2008; Khedri et al., 2014; Dawoodi, 2014). In previous studies, *S. digitata, S. marshalli,* and *S. labiatopapillosa* have been isolated from cattle and buffaloes from different regions of Iran, while in our study, *S. labiatopapillosa* was the only isolated *Setaria* species from cattle of southwest of Iran. The difference between the findings may be due to differences in climate, the presence of a suitable intermediate hosts and livestock management systems in various regions.

Setariosis in buffaloes has been reported from

different countries such as India and Iran. The prevalence rate of infection varies from 0.9% to 54% (Dawoodi, 2014; Siddiqui et al., 1996; Patnaik, 1989; Chauhan and Pande, 1980) but in this study, no infection with *Setaria* parasite was observed in buffaloes. The reasons for the lack of setarial infection in buffaloes in this area have not been clarified yet. It may be related to presence of genetic resistance, differences in the structure of the skin and the absence of specific intermediate host for development and transmitting the parasite to the buffaloes. To strengthen one of the previous assumptions it has been found that buffaloes have genetic resistance to some internal and external parasitic infection such as tick infestation, fascioliasis and theileriosis (FAO, 2007).

The present study provides detailed information about the ultrastructures of *S. labiatopapillosa*. Conventionally, the light microscope is used to identify the *Setaria* spp. but, sometimes due to the low-resolution images, an accurate identification of some species (i.e., *S. labiatopapillosa* and *S. marshalli*) becomes difficult and leads to a misdiagnosis; on the contrary, ultrastructural images prepared by the SEM are of high quality and facilitates the identification of the *Setaria* spp. (Almeida et al., 1991; Ronghang and Roy, 2013; Kumar and Kumar, 2016). Some morphological features such as amphid, ventral bands, patterns of cloacal papillae in male worms and the genital pore in female worms were clearly visible with SEM. These structures were not detectable with light microscope. It should be noted that SEM (Scanning Electron Micrographs) of samples which were prepared with the osmium tetroxide lacked adequate quality sometimes were collapsed and some structures such as amphids were not detectable while these problems did not exist in samples that were only fixed with 5% glutaraldehyde. According to our results, it seems that the use of osmium tetroxide is not suitable for the preparation of *Setaria* spp.

Different studies have shown that larval stages of filarial species usually cannot be differentiating by classical morphology. Analysis of genetic markers can also be reliable for the accurate differentiation of *Setaria* spp. and the determination of genetic diversity in parasites originated from different geographical areas. In the present study, molecular characteristics and genetic variations of *Setaria* spp. isolated from cattle and originated from southwestern Iran were determined by PCR-sequencing of the 12S rDNA and *cox1* genes. In fact, previous studies have shown that these sequences provide reliable genetic markers for the accurate differentiation and identification of *Setaria* spp. The analyses confirmed that all the sequences from the cattle and localities are identical to those of previously submitted to GenBnak for *S. labiatopapillosa*. These results indicated that this species is the main *Setaria* species involved in the spread of Setariosis in southwestern Iran.

The 12S rDNA sequences of *S. labiatopapillosa* obtained in this study showed nucleotide variations in 53 positions. The comparisons of all obsereved *cox1* sequences with related sequences of *cox1* of *S. labiatopapillosa* from other geographical areas showed lower nucleotide differences in compared with 12S rDNA. Yatawara et al. (2007) noted that mitochondrial *cox1* and 12S rDNA genes are conserved in *S. digitate*. They also mentioned that *S. digitata* and *S. labiatopapillosa* appear to be sister species.

The phylogenetic tree constructed using *cox1* and 12S rDNA genes of several other filarial nematodes showed that the Khuzestan isolates share a common branch with *S. labiatopapillosa.* Low intra-specific variation was observed in 12S rDNA but not in *cox1*. In fact, groups of multiple closely related genotypes of *S. labiatopapillosa* obtained in the present study are broadly sympatric. Such pattern is expected for species with high gene flow, whose populations have not been sundered by long-term biogeographic barriers.

The genetic characterization of *Setaria* Spp. present in southwestern Iran is useful to achieve the basic information necessary for the field control of this parasite and may have implications for the diagnosis and control of the disease. To better understand the genetic variability and population genetic structure of *Setaria* spp. in Iran and in oth-

er neighboring areas a wide range of isolates from different hosts and geographical localities and the use of more variable genetic markers are needed

In conclusion, our results showed that infection of cattle with *S. labiatopapillosa* is common in southwestern Iran while buffalo may be free from filarioid nematodes. From the above findings, it can be proved that DNA sequences of 12S rDNA and *cox1* genes are useful molecular tools for accurate identification of *Setaria* species. Further studies are necessary in order to recognizing the molecular and morphological characteristics of other filarial species infecting cattle in Iran, its vectors and possible prevention.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST STATEMENT

The authors confirm that there is no conflict of interest.

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Research article
Ερευνητικό άρθρο

# The Phenotype Variability, of the Racka Sheep in the Republic of Serbia

**I. Pihler[1*], J. Ćirić[1], D. Kučević[1], S. Dragin[1], M. Al-Hasant[2], M. Šaran[1], B. Zarubica[1], I. A. Tsakmakidis[3]**

[1]*Department of Animal Science, Faculty of Agriculture, University of Novi Sad*

[2]*National project Director Mainstreaming Biodiversity Conservation in Tourism sector development in Jordan UNDP*

[3]*Faculty of Health Sciences, School of Veterinary Medicine, Aristotle University of Thessaloniki*

**ABSTRACT.** The intensification of sheep production, by permanent genetic selection and the development of breeding technology, has led to the creation of highly productive sheep breeds. In this way, many highly productive breeds were created which could demonstrate their high production potentials only under perfect conditions of nutrition, accommodation and care. Preservation of indigenous breeds is of great importance in order to protect and safeguard those breeds and, in this way, it is possible to restore some of the characteristics that are lost during intensive selection, which are mostly related to resistance. The Racka sheep (Serbian: Vitoroga žuja) is considered to be an autochthonous breed and a genetic resource in the Republic of Serbia. As a primitive breed with low productivity, it offers no economic profitability and, thus, there is no great interest in its breeding. According to the FAO data from 2008-2014, the number of these sheep ranges from 500 to 1000. The objective of this study was to determine the phenotypic variability and to assess the external measurements of the Racka sheep. One-hundred fifty Racka breed ewes were included in this study. The effects of three farms on the phenotypic characteristics and their body indexes were calculated. The significance of the research is reflected in the advancement of this breed and in the assessment of the possibilities of selection work in these herds.

***Keywords**:* The Racka sheep, phenotypic characteristics, variability of characteristics.

*Corresponding Author:*
Ivan Pihler, Department of Animal Science, Faculty of Agriculture, University of Novi Sad, Square Dositeja Obradovica 8, 21000 Novi Sad
E-mail address: ivan.pihler@stocarstvo.edu.rs

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## INTRODUCTION

The indigenous sheep breeds have originated in one geographical area, and they have been adapted to the specific living conditions. Most commonly, those are primitive sheep. A breed becomes a genetic resource if the number of sheep of that breed is below 1000. Genetic resources are of fundamental importance, and so is their conservation. Although"Vitoroga žuja", or the Racka sheep, belongs to the group of animal genetic resources of the Republic of Serbia, indigenous breeds are also an important source of genetic potential for future work in livestock husbandry (Drobnjak, 2012).

The research work of Savić et al., (2014) found a favorable ratio of fatty acids and good sensory properties of lamb meat in the age of 100 days. The milk of Racka sheep has a good chemical composition (Viturro et al., 2015), suitable for the production of traditional dairy products.

The population of the autochthonous breeds is getting smaller and there are often cases of inbreeding, which increases the frequency of homozygosity within the population and creates a real risk of losing certain genes (Drobnjak, 2012).

The Racka sheep is one of the many strains of the pramenka sheep. It is considered an autochthonous breed and a genetic resource in the Republic of Serbia. The largest population is found in the Banat region of Vojvodina. According to Gáspárdy (2010), the Racka sheep (in Germany "Zackel-Schaf"), is a group of sheep that relates to sheep of a similar form, which were kept in regions where they were originally found - "Vlaska" (Slovakia), "Tschekel" (Ukraine), "Turcana" (Romania), "Racka" (Hungary), "Vlasko-Vitoroga" (Serbia).

It appears in two variants: white (yellowish-brown) and black. It is used in the production of meat, milk and wool (combined type). Its phenotype is characterized by their long horns - ewes (~ 20 cm) and rams (~ 50 cm). The horns are grayish to yellow and they are elongated and spirally twisted. The quality of the wool is very low and its diameter ranges from 12-40 microns. The body weight of the ewes is about 40 kg and the rams weigh about 60 kg. The milk yield after lactation is small and it ranges between 50 and 180 l (Nagy, 2006).

**Figure 1.** Ewes of the Racka sheep that were used in this research

**Figure 2.** A Racka sheep ram of the researched herd

As a low productive and primitive breed, there is no economic profitability to it and there is no great interest in its breeding. Another factor which causes a decrease in the number of sheep are dwindling pasture areas. Unfortunately, the breeders often cross this sheep breed with other breeds of higher productivity in order to achieve greater growth, which threatens the survival of the Racka sheep.

The population of the Racka sheep is small in our area. According to the FAO data from 2008-2014, the number of heads ranges from 500 to 1000. In the Republic of Serbia, the Racka sheep is considered an endangered autochthonous breed. According to the data supplied by the Main Breeding Organization of the Department of Animal Husbandry, Novi Sad, there are around 900 sheep under controlled production (Professional report and results of controls of implemented breeding programs in APV for 2017).

The first step in preserving this genetic resource is to determine the phenotypic breed characteristic and its similarity to the same or similar breeds in the region. The most recent analysis of the Racka sheep was done in Hungary, where the largest population of this breed is located. In Serbia, the region of the South Pannonian Plain, extending to the south of the country, the Racka sheep is a common breed, but it has never been sufficiently described, and so far there has been no serious and detailed measurement and standardization of the exterior features of this race.

Taking into account the above mentioned, the aim of the study was to create a valid set of data of exterior measurements, which may be used as a base for determination (and further research) of the genetic structure of the breed.

## MATERIALS AND METHODS

The research was conducted on the population of the Racka sheep located in the territory of the Autonomous Province of Vojvodina. All sheep were in the pasture-based rearing system. A total of 150 ewes were measured on three different farms.

All sheep were between first and third month of pregnancy. The farms use extensive breeding and pastures as a feed source throughout the year. The measurement was conducted by a single person, with an assistant's help. The influence of the evaluator is excluded in this study.

The measurement of exterior dimensions is important in order to gather data on the corpulence of each animal. The measurements were made using a height measuring stick and tape. Each sheep was measured on a flat surface. Measures were taken from the left side of the animal. The collected data were placed in ratio formula, in order to make the calculations comparable. In this way, 10 body indexes were obtained.

The measurements that were used include:

- The height of the wither - it was measured with a measuring stick, from the surface behind the lower rear edge of the foreleg cloven hoof, vertically to the highest edge of the wither,

- The height of the back - vertically from the surface to the highest point of the back line of the last chest vertebra,

- The height of the loin - vertically from the surface to the highest point of the loin,

- The body length - horizontally from the shoulder joint to the rear point of the rump

- The depth of the chest - from the chest bone to the vertical back line behind the wither

- The length of the chest - from the shoulder joint to the last rib

- The width of the chest - the width in the line of the third vertebra

- The circumference of the chest - measured with a tape measure, behind the shoulder blades

- The width of the pelvis - from the external of one side to the external of the other side of the rump

- The circumference of the shin - it was measured with a tape on the thinnest part of the shin

- The width of the forehead - the width of the widest part of the forehead

- The length of the head - from the upper part of the forehead bone to the nasal bone

- The length of the horns - from the root to the end of the horn, twisting a tape on the external horn groove

- The length of the ears - from the root to the end of central exterior line of the ears

**Figure 3.** External dimensions - the wither height, the back height and the loin height, which have been used during the measurement.

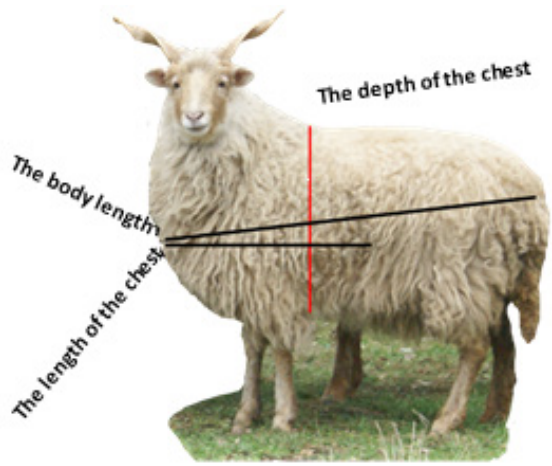


**Figure 4.** External dimensions - the chest length, the body and the chest length which have been used during the measurement.

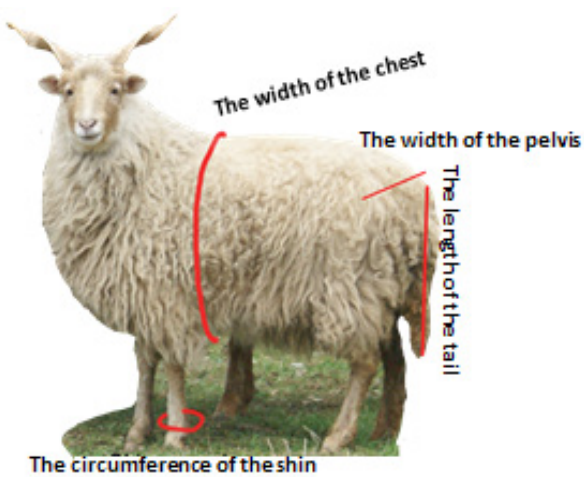


**Figure 5.** External dimensions - the circumference of the chest, the pelvis width and the circumference of the shin, which have been used during the measurement.

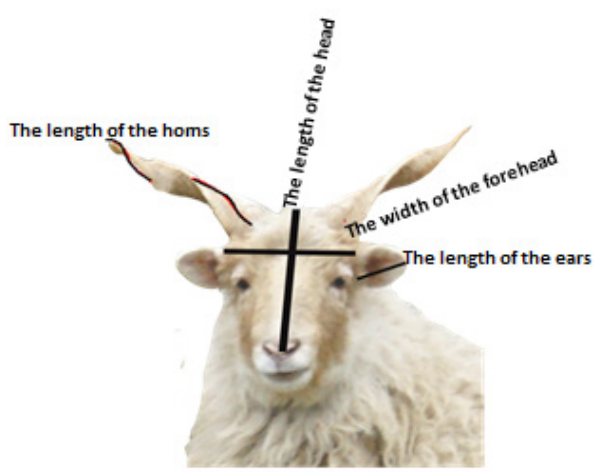


**Figure 6.** External dimensions - the horn length, the length of the head and the length of the ears, which have been used during the measurement.

The calculated indexes:

The body form index represents the ratio between the body length and the wither height

$$Index = \frac{\text{body length}}{\text{wither height}} \text{ X } 100$$

The chest index represents the ratio between the chest width and its depth

$$Index = \frac{\text{chest width}}{\text{chest depth}} \text{ X } 100$$

The chest depth index represents the ratio of the chest width and the wither height

$$Index = \frac{\text{chest depth}}{\text{wither height}} \text{ X } 100$$

The body compactness index represents the ratio between the circumference of the chest and the body length

$$Index = \frac{\text{chest girth}}{\text{body length}} \text{ X } 100$$

The mass index represents the ratio between the circumference of the chest and the height of the wither

$$Index = \frac{\text{chest girth}}{\text{wither height}} \text{ X } 100$$

The partition index represents the ratio between the height of the loins and the height of the wither

$$Index = \frac{\text{loins height}}{\text{wither height}} \text{ X } 100$$

The pelvis-chest index represents the ratio between the width of the chest and the width of the pelvis

$$Index = \frac{\text{chest width}}{\text{pelvis width}} \text{ X } 100$$

The leg length index represents the ratio between the difference of the wither height and the chest depth and the wither height

$$Index = \frac{\text{wither height - chest depth}}{\text{wither height}} \text{ X } 100$$

The gauntness index represents the ratio between the circumference of the shin and the height of the wither

$$Index = \frac{\text{circumference of the skin}}{\text{wither height}} \text{ X } 100$$

The forehead width index represents the ratio of the forehead width and its length

$$Index = \frac{\text{forehead width}}{\text{forehead length}} \text{ X } 100$$

Statistical processing of measured and calculated data, such as the average (mean) value ($\bar{x}$), minimum and maximum were processed in Microsoft Excel 10 software package, while standard deviation (SD), coefficient of variability (CV), standard error (S.E.), and determination of the location's influence on certain characteristics (analysis of variance) were calculated in Statistica 13.3. program.

## RESULTS

Table 1 shows descriptive statistical analysis for the entire population used during the research. In table 1 we can see that traits horn length and tail length have highest values of coefficient of variability, 16.86 and 13.37, respectively.

In order to determine the influence of the location (farm) on some of the external dimensions, a variance analysis was performed. The results are shown in Table 2.

**Table 1.** Phenotype Variability of exterior body traits for Racka sheep population

| Body traits | Descriptive statistics for all farms | | | | | |
|---|---|---|---|---|---|---|
| | N | $\bar{x}$ (cm) | SD | CV | S.E. | Min - Max |
| Wither height | 150 | 64.31 | 3.78 | 5.87 | 0.31 | 54.0 to 74.0 |
| Back height | 150 | 64.69 | 3.95 | 6.10 | 0.32 | 54.0 to 79.0 |
| Loin height | 150 | 64.25 | 3.92 | 6.10 | 0.32 | 54.0 to 73.0 |
| Body length | 150 | 69.56 | 2.35 | 3.38 | 0.19 | 60.0 to 75.0 |
| Chest length | 150 | 37.97 | 2.31 | 6.08 | 0.19 | 32.0 to 46.0 |
| Chest depth | 150 | 30.26 | 2.49 | 8.24 | 0.20 | 21.0 to 38.0 |
| Chest width | 150 | 18.89 | 1.70 | 9.01 | 0.14 | 14.0 to 22.0 |
| Pelvis width | 150 | 19.82 | 1.47 | 7.44 | 0.12 | 16.0 to 25.0 |
| Chest girth | 150 | 85.25 | 3.83 | 4.50 | 0.31 | 77.0 to 96.0 |
| Shin circumference | 150 | 7.99 | 0.44 | 5.45 | 0.04 | 7.0 to 9.5 |
| Tail length | 150 | 38.01 | 5.08 | 13.37 | 0.41 | 17.0 to 50.0 |
| Head length | 150 | 19.25 | 0.66 | 3.43 | 0.05 | 16.0 to 21.0 |
| Forehead width | 150 | 10.47 | 0.68 | 6.54 | 0.06 | 9.0 to 13.0 |
| Horn length | 150 | 26.72 | 4.50 | 16.86 | 0.37 | 12.0 to 38.0 |
| Ears length | 150 | 10.50 | 0,97 | 9.20 | 0.08 | 9,0 to 13.0 |

*$\bar{x}$- average value, SD - Standard deviation, CV - Coefficient of Variability, S.E. - Standard Error*

**Table 2.** The results of variance analysis, for stastistical differences of the measured external dimensions of the Racka sheep

| | Variance analysis for all dimensions | | | |
|---|---|---|---|---|
| | SS | MS | F | P |
| Wither height | 975.29 | 487.65 | 62.28 | 0.00** |
| Back height | 1015.05 | 507.53 | 57.07 | 0.00** |
| Loin height | 1022.01 | 511.01 | 59.13 | 0.00** |
| Body length | 6.84 | 3.42 | 0.62 | 0.54 |
| Chest length | 4.89 | 2.45 | 0.46 | 0.63 |
| Chest depth | 124.36 | 62.18 | 11.39 | 0.00** |
| Chest length | 58.97 | 29.49 | 11.65 | 0.00** |
| Pelvis width | 5.32 | 2.66 | 1.23 | 0.30 |
| Chest girth | 440.09 | 220.05 | 18.48 | 0.00** |
| Shin circumference | 0.63 | 0.32 | 1.68 | 0.19 |
| Tail length | 54.97 | 27.49 | 1.07 | 0.35 |
| Head length | 2.41 | 1.21 | 2.84 | 0.06 |
| Forehead width | 2.29 | 1.15 | 2.50 | 0.09 |
| Horn length | 574.24 | 287.12 | 17.23 | 0.00** |
| Ears length | 24.96 | 12.48 | 16.09 | 0.00** |

*** Highly significant statistical difference (p<0.01), SS - Square sum, MS -Mean sum, f - f value*

Statistical differences (p<0.01) of the Racka sheep from different locations were noticed for 8 external measures. Those characteristics are underlined in Table 2.

Indexes are absolute values of a measurement relative to some other body measurement, expressed as a percentage. These indexes serve to determine the proportions of the animal body and for a more precise comparison of the individual's development, (Ćinkulov et al., 2003). By calculating the external measurements, the obtained indexes were statistically analyzed.

Table 3 shows the average values of calculated indexes. Based on the coefficient of variability, we can see that the values of individual indexes are very variable. The form index and the chest index had the highest degree of variability. Comparisons between the farms were made by the variance analysis, as shown in Table 4. Statistical differences (p<0.01) of the Racka sheep from different locations were noticed for 6 body indexes.

**Table 3.** Phenotype Variability of exterior body indexes for Racka sheep population from all farms.

| Body traits | Descriptive statistics for all farms | | | | | |
|---|---|---|---|---|---|---|
| | N | $\bar{x}$ | SD | CV | S.E. | Min - Max |
| The form index | 150 | 108.49 | 6.74 | 6.21 | 0.55 | 85.71 - 131.48 |
| The chest index | 150 | 62.75 | 6.88 | 10.96 | 0.56 | 45.16 - 86.36 |
| The chest depth index | 150 | 47.11 | 3.71 | 7.88 | 0.30 | 33.33 - 55.88 |
| The body compactness index | 150 | 122.67 | 6.39 | 5.21 | 0.52 | 105.48 - 142.42 |
| The mass index | 150 | 132.86 | 7.44 | 5.60 | 0.61 | 117.81 - 150.85 |
| The partition index | 150 | 99.94 | 3.03 | 3.03 | 0.25 | 90.91 - 115.00 |
| The pelvis-chest index | 150 | 95.53 | 8.49 | 8.89 | 0.69 | 73.68 - 125.00 |
| The leg length index | 150 | 52.89 | 3.71 | 7.02 | 0.30 | 44.12 - 66.67 |
| The gauntness index | 150 | 12.46 | 0.92 | 7.39 | 0.08 | 10.00 - 14.81 |
| The forehead width index | 150 | 54.44 | 4.26 | 7.82 | 0.35 | 45.00 - 81.25 |

*$\bar{x}$ - the average value, SD - Standard Deviation, $\sigma^2$ - Variance, CV - Coefficient of Variability, S.E. - Standard Error*

**Table 4.** The variance analysis, for statistical differences of the body indexes of the Racka sheep.

| | The variance analysis | | | |
|---|---|---|---|---|
| | SS | MS | F | p |
| The form index | 2987.59 | 1493.79 | 58.07 | 0.00** |
| The chest index | 329.76 | 164.88 | 3.61 | 0.03** |
| The chest depth index | 32.64 | 16.32 | 1.19 | 0.31 |
| The body compactness index | 1005.89 | 502.94 | 14.55 | 0.00** |
| The mass index | 1888.05 | 944.03 | 21.85 | 0.00** |
| The partition index | 8.37 | 4.18 | 0.45 | 0.64 |
| The pelvis-chest index | 1741.72 | 870.86 | 14.22 | 0.00** |
| The leg length index | 32.64 | 16.32 | 1.19 | 0.31 |
| The gauntness index | 24.34 | 12.17 | 17.54 | 0.00** |
| The forehead width index | 29.15 | 14.57 | 0.80 | 0.45 |

*** Highly significant statistical difference; SS - Square sum MS - Mean sum f - f value*

## DISCUSSION

Small differences in the average measurements of wither height (64.31), height of the back (64.69) and height of the loin (64.25) (Table 1) show the flat backline of the Racka sheep population in Serbia. The observed average wither height is lower than that of the Hungarian Racka ewes as shown in the research paper of Nagy (2006), 66,97 for black and 68.20 for white sheep, but higher than in the ewes from a research carried out in Banat, which were 54,24 cm high (Savić et al., 2013).

Statistically significant differences in measured body dimensions (Table 2) between the three observed sites occurred in 8 of the 15 observed characteristics. Statistically significant differences are related to body dimensions, which describe the animals' form. The most important are the height of the withers, the height of the

back, the height of the loins as well as the width and the circumference of the chest. This indicates that the herds exhibit no uniformity either in their physical fitness or constitution. Such differences occur due to unequal keeping and nursing conditions (primarily because of unequal nutrition) (Ćinkulov et al., 2003).

The length of the head and the ears, and the width of the forehead describe the Racka sheep as a sheep with a small and narrow head. The width of the forehead, the length of the head and the circumference of the shin did not show statistically significant differences. These measurements are one of the important racial features that are hardly variable (Krajinović, 2006).

Statistically significant differences were not found in the body length of animals in the observed locations. This trait ranged from 60.00 to 75.00 cm, while the mean value of the body length was 69.56 cm. Similar results for this feature were also indicated by Savić et al. (2013). But they are shorter in comparison to the sheep from Hungary, with average length of 73,36 and 70.77 (Bodó, 1994). The length of the body is a measurement that is very important in the phenotypic assessment and selection of animals used for further reproduction based on phenotype. A statistically significant difference did not occur in the length of the chest, as expected, given the fact that it is a measurement that is linearly related to the length of the body (Krajinović, 2006).

Statistically significant differences occurred in 6 of 10 analyzed indexes observed in these locations. This is expected, given the fact that a larger number of external measurements also showed statistically significant differences. The chest index had a statistically significant difference on the observed sites, while the chest depth index did not have statistically significant differences.

On the basis of the average index of the body compactness, it can be seen that the depth of the chest had a higher value than the length of the body and this index shows a great degree of variability since there are statistically significant differences on the observed sites.

The mass index also showed a statistically significant difference between the observed sites. The mass index of our indigenous breeds is always closer to 100% (Ćinkulov et al., 2003). The determined values of the mass index were higher than those (125.6 and 127.6) which Bodó (1994) found in Hungary. The reason for the differences between the populations in Serbia and Hungary may be geographical isolation and different genetic makeup. According to Dudu et al., (2016) starting from 1960, Hungary imported sheep from Romania and afterwards the breed was exposed to an intensive process of selection and conservation.

The partition index did not have statistically significant differences. The height of the wither (64.25 cm) and the loin (64.31 cm) of the whole population was very similar (small coefficient of variation), which is the consequence of a uniformed partition index. The value of this index was around 100, which is highly desirable.

A large statistically significant difference was found in the pelvis-chest index, while the indexes of the leg length and forehead width did not have statistically significant differences between the farms.

## CONCLUSIONS

By measuring external measurements of, practically, one quarter of the Racka sheep population in the Republic of Serbia, it can be concluded that the highest variability of the characteristics occurs when it comes to the form of each individual sheep, while the measurements that are part of the important breed characteristics did not show important statistical differences. The influence of exterior factors, such as the nutrition patterns, breeding technology and the keeping conditions of the animals, above all, represent the causes of the differences. The display of the phenotypic characteristics of the Racka sheep has opened up the possibility for further research of this breed, which has lived in the Pannonian plain for more than 1,000 years. The next logical step is to determine genetic differences, and similarities within different strains of this breed in the region, and to create a detailed regional preservation plan.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Analyzing risk factors for lumpy skin disease by a geographic information system (GIS) in Turkey

**O.B. Ince[1*], T. Türk[2]**

[1] *Province Directorate, Ministry of Agriculture and Forestry, Afyonkarahisar, Turkey*

[2] *Department of Geomatics Engineering, Cumhuriyet University, Sivas, Turkey*

**ABSTRACT.** Lumpy skin disease (LSD) is caused by the virus of the same name and has major economic impacts on cattle breeding. In Turkey, frequent cases of cattle LSD have been reported over the last years. The present study aimed to analyze potential risk factors for LSD and provide information for controlling the spread of infectious diseases by a geographic information system (GIS). The research included cross-sectional and retrospective studies with active disease follow-up and semi-structured interviews (SSI) from August 2013 to December 2014 in Turkey. Potential risk factors for LSD were evaluated based on environmental conditions and provincial demographic and epidemiological data. Of the total of 562 observed animals, 27.22% and 2.67% of cattle were sick and died due to LSD, respectively. The morbidity rate was 26.04% in mixed and 38.18% in local breeds. The animal-level prevalence significantly differed among animals of different age, sex, and with different vaccination status ($P<0.05$). It was more serious in younger animals and females and during drier weather conditions. A trend of seasonality was observed in LSD occurrence. Significant risk factors affecting the prevalence of LSD were proximity to the southern border of Turkey, animal movements, and animal markets. In this process, geographical query, analysis, and thematic map production were performed by GIS.

***Keywords:*** LSD, Epidemiology, Risk factors, GIS, Animal Movement

*Corresponding Author:*
O.B.Ince, Province Directorate, Ministry of Agriculture and Forestry, 03100, Afyonkarahisar, Turkey
E-mail address: incebaris@gmail.com, omerbaris.ince@tarimorman.gov.tr

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## INTRODUCTION

Lumpy skin disease (LSD) is a poxviral infection of cattle caused by lumpy skin disease virus of the genus Capripoxvirus. It is a Neethling virus. This pox virus causes an acute to a sub-acute systemic disease characterized by mild to severe symptoms including fever, nodules in the skin, mucous membranes, and internal organs, and sometimes death (Davies, 1982). In general, mortality is low (1–3%), but up to 75% mortality has been reported (Babiuk et al., 2008). LSD is associated with significant production losses. It is therefore defined as a notifiable disease by the World Organization for Animal Health (OIE, 2000). LSD is related to various factors that cause significant financial losses on a national scale: restrictions on the global trade of live animals and their products (Rich and Perry, 2011, Tuppurainen and Oura, 2012).

LSD infection has a wide distribution in most African countries (Niger, Sudan, Uganda, Ethiopia, Somalia, etc.) and has recently entered into the Middle East (EFSA, 2015). In this study, we assess risk factors in the spread of LSD in Turkey from the Middle East as well as the risks of further spread. Moreover, the present study aimed to analyze potential risk factors for LSD. We address the epidemiology of LSD, its mechanisms of transmission, the potential role of risk factors in reducing spread, and currently available control strategies. The first findings on LSD in Turkey were reported in mid-2013, and potential LSD vectors, such as animal movement (Alemayehu et al., 2013) and blood-sucking insects (Tuppurainen et al., 2011), were identified. Despite this knowledge, the epidemiological status of cattle LSD in Turkey and its risk factors are not completely understood. This study aims to reveal LSD-infected cattle farm areas and analyze risk factors such as cattle sex, increasing age, cattle breed, season, and vaccination using a Geographic Information System (GIS).

## MATERIAL AND METHODS

### Epidemiological data and GIS

The study was conducted in nine Turkish provinces: Kahramanmaraş, Batman, Hakkari, Malatya, Adıyaman, Osmaniye, Hatay, Sivas, and Adana (Fig.1). These provinces are located along the southern border of Turkey and have a large number of family-run small- and medium-sized dairy farms. Data on LSD outbreaks, farms, and cattle movements were obtained from the animal registration system and the World Organization for Animal Health (OIE). The assessment was based on a combination of active disease follow-up and questionnaire and retrospective data collection that focused on 70 pastoral and agro-pastoral farms. The study was conducted with a cross-sectional and retrospective design which employed active disease follow-up and semi-structured interviews (SSI) from August 2013 to December 2014. SSIs were used to generate information about the prevalence and history of LSD, bio-data for individual animals, and disease-related losses including death and abortion. Cattle owners were selected randomly based on the willingness to complete the questionnaire. Questionnaire interviews were carried out in these provinces face-to-face. The sample included 562 animals of different ages, sexes, and breeds (Local breeds: Eastern Anatolian Red (25), Southeast Anatolian Red (30); Mixed breeds: Holstein-Friesian (215), Brown Swiss (227), and Fleckvieh (65)). All farms were reported to have had LSD outbreaks. These provinces have a mean annual minimum and maximum temperature of -7°C and 41.3°C, respectively.

A GIS is an information system that collects and stores data for queries, documenting (producing maps and creating tables), and spatial analysis. It has been used in various fields by many researchers, including animal health studies (Michel et al., 2002, Soumare et al., 2007, Tuma et al., 2007, Carpenter, 2011). A user interface program ensures interaction between the user and the computer. Although many processes are performed for complex spatial analysis and queries in GIS software, these processes can be executed by means of pressing a button in developed user interface programs (Türk et al., 2012). Thus, the program can be used easily without manuals, and effective and efficient results can be produced (Türk et al., 2012, Türk, 2013). Due to the fact that maps are a visual communication tool, GIS offers a means to interpret and understand the data better. For example, you cannot know the border of provinces or countries without thematic maps. Consequently, GIS users have got the possibility to access information easily via query and to analyze spatial data by GIS. In this study, a GIS-based system was created to query, analyze and document data related to animal health, so that the related institutions and users could use it. All data were integrated into the GIS environment. User interface programs were developed (Fig. 2).

**Fig 1.** The study area

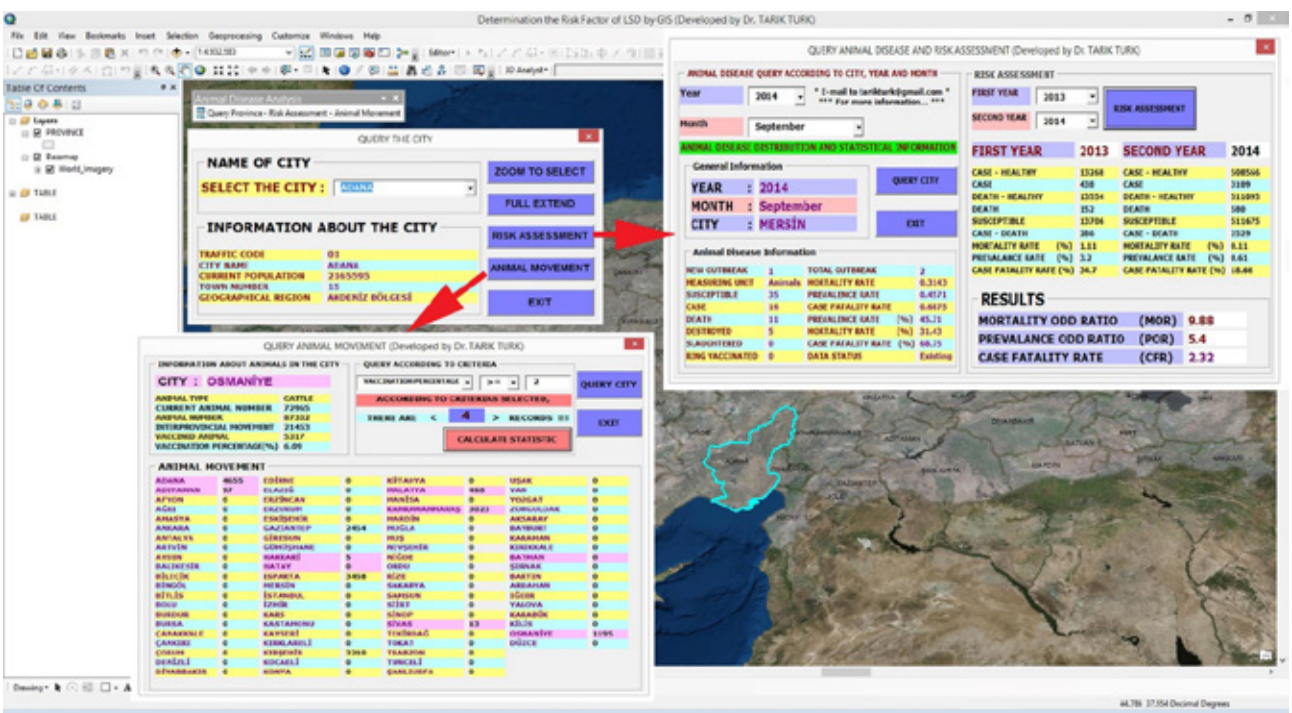


**Fig 2.** The GIS based system

**Data management and analysis**

Data were entered and stored in Microsoft Excel spreadsheets. The univariate association of potential risk factors with the animal-level prevalence of LSD and statistical significance were evaluated using the Chi-square ($\chi 2$) test. Logistic regression analysis was used to compute the strength of contribution of the risk factors to LSD occurrence. Data were analyzed in R version 3.1.2 (R Core Team, 2014) and SPSS V21. The Odds ratio (OR) was calculated for each risk factor for animals sick with LSD. In all the analyses, confidence levels at 95% were calculated, and a value of $P<0.05$ was accepted as statistically significant. The OR was calculated for the risk factors and animals sick with the disease to determine the degree of association between the risk factors and the disease. Descriptive statistics such as prevalence were used to calculate rates by dividing the number of animals sick with LSD by the total number of animals at risk. Finally, data were integrated with ESRI ArcGIS 10.1 GIS software, which was also used to query and analyze the data. Variables with $P < 0.25$ were shortlisted to consider in the final multivariable logistic regression analysis. The model was built stepwise by forward selection, adding shortlisted factors and removing the factors when $P > 0.05$. The effects of interactions among significant variables in the final model were tested by pairwise interactions in the multivariable logistic models. The model fitness was assessed by the likelihood-ratio test. The mortality odds ratio, prevalence odds ratio, and case fatality rate were determined by comparing cases/death/healthy animal number in different periods. These processes are performed automatically by the user interface program developed in the GIS-based system.

**Table 1.** Morbidity, mortality, case fatality rates and odds ratio of LSD for each investigated risk factors in Turkey between 2013 and 2014.

| Breed | Variables | Factor Levels | No. at risk | No. of sick | No. of death | Morbidity rate | Mortality rate | Case fatality rate | Odds Ratio | 95%CI | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mixed | **Age** | <24 month * | 151 | 93 | 10 | 61.59 | 6.62 | 10.75 | | | |
| | | 24-48 month | 174 | 27 | 3 | 15.52 | 1.72 | 11.11 | 0.11 | 0.06-0.19 | 0.001 |
| | | >48 month | 182 | 12 | 0 | 6.59 | | | 0.04 | 0.02-0.08 | 0.001 |
| | **Sex** | Female* | 401 | 120 | 9 | 29.93 | 2.24 | 7.5 | | | |
| | | Male | 106 | 12 | 4 | 11.32 | 3.77 | 33.33 | 3.35 | 1.76-6.32 | 0.001 |
| | Vaccination | Vaccinated* | 484 | 121 | 12 | 25.00 | 2.48 | 9.92 | | | |
| | | Non-Vaccinated | 23 | 11 | 1 | 47.83 | 4.35 | 9.09 | 0.36 | 0.15-0.84 | 0,01 |
| | **Season** | Autumn* | 121 | 35 | 0 | 28.93 | | | | | |
| | | Winter | 82 | 24 | 0 | 29.27 | | | 1.01 | 0.54-1.88 | 0.95 |
| | | Spring | 30 | 4 | 0 | 13.33 | | | 0.37 | 0.12-1.16 | 0.08 |
| | | Summer | 274 | 69 | 13 | 25.18 | 4.74 | 18.84 | 0.82 | 0.51-1.33 | 0.43 |
| | | Total | 507 | 132 | 13 | 26.04 | 2.56 | 9.85 | | | |
| Local | Age | <24 month * | 7 | 3 | 2 | 42.86 | 28.57 | 66.67 | | | |
| | | 24-48 month | 17 | 5 | 0 | 29.41 | | | 0.55 | 0.08-3.44 | 0.42 |
| | | >48 month | 31 | 13 | 0 | 41.94 | | | 0.96 | 0.18-5.05 | 0.64 |
| | **Sex** | Female* | 27 | 15 | 2 | 55.56 | 7.41 | 13.33 | | | |
| | | Male | 28 | 6 | 0 | 21.43 | | | 4.58 | 1.4-14.90 | 0.009 |
| | Vaccination | Vaccinated* | 24 | 15 | 2 | 62.50 | 8.33 | 13.33 | | | |
| | | Non-Vaccinated | 31 | 6 | 0 | 19.35 | | | 6.94 | 2.05-23.44 | 0.001 |
| | **Season** | Autumn* | 23 | 8 | 0 | 34.78 | | | | | |
| | | Winter | 2 | 1 | 0 | 50.00 | | | 1.87 | 0.10-34.13 | 0.59 |
| | | Spring | 8 | 2 | 0 | 25.00 | | | 0.62 | 0.10-3.84 | 0.48 |
| | | Summer | 22 | 10 | 2 | 45.45 | 9.09 | 20.00 | 1.56 | 0.47-5.18 | 0.46 |
| | | **Total** | 55 | 21 | 2 | 38.18 | 3.64 | 9.52 | | | |

*ref:<24-month, female, vaccinated, autumn

## RESULTS

All farms were within pastoral and agro-pastoral areas. The average herd size was 15 and ranged from 5 to 42 animals. A mixture of farming systems was practiced in each province: 30% of farms in Hakkari and Batman provinces were pastoral; 40% of farms in Osmaniye, Adana, and Hatay provinces were agro-pastoral.

Only 54 out of 562 animals were not vaccinated against LSD. It was recorded that 9.61% (54/562) of animals were affected by the disease and 2.67% (15/562) eventually died of it. Most infected animals (27.22% or 153/562) and all dead animals, except for one, were vaccinated against LSD. Breed-specific morbidity, mortality, and fatality rates were analyzed. The morbidity rate for mixed and local breeds was 26.04% (132/507) and 38.18% (21/55), respectively. The mortality and fatality rates were 2.56%, 9.85% (mixed breeds) and 3.64%, 9.52% (local breeds), respectively. In mixed breeds, the morbidity rate was 25.00% (121/484) in vaccinated and 47.82% (11/23) in non-vaccinated cattle ($P<0.05$). In local breeds, the morbidity rate was 62.50% (15/24) in vaccinated and 19.35% (6/31) in non-vaccinated cattle ($P<0.05$). In mixed breeds, the morbidity and mortality rates decreased with age. For animals under 24 months of age, they were 61.59%, 6.62%, for those aged 24–48 months, they were 15.52%, 1.72%, and in adults over 48 months of age, the morbidity rate was 6.59%, respectively ($P<0.05$). The detailed results are presented in Table 1.

A trend of seasonal distribution of LSD was observed in 2014. The month of August had the highest number of outbreak reports. Outbreaks increased from June to October 2014. The multivariable logistic regression analysis revealed that age, sex, and vaccination status were risk factors associated with the occurrence of LSD. By using the Hosmer-Lemeshow goodness-of-fit statistics, the Chi-square value was calculated as 2.15, the related significance value as 1.000, the model's Pseudo $R^2$ value as 0.725, and the model's overall classification ratio as 85.4%. According to the multivariable logistic regression analysis results, age and sex of animals showed a significant association with LSD. The results are presented in Table 2.

The disease was identified for the first time in Kahramanmaraş province on August 6, 2013, and spread to Batman, Hakkari, Malatya, Adıyaman, Osmaniye, Hatay, Sivas, and Adana provinces. As of June 17, 2014, it has spread to Mersin, Kayseri, and Şanlıurfa provinces. Within the framework of the EU and national legislation, there is a struggle against the disease. There were 91 outbreaks in nine provinces over the nine-month period from August 6, 2013, to May 8, 2014. Eighty-eight outbreaks (96.70%) occurred in seven provinces: Kahramanmaraş, Malatya, Sivas, Adıyaman, Osmaniye, Hatay, and Adana. The remaining three outbreaks (3.30%) occurred in two provinces: Batman and Hakkari. Eighteen outbreaks (19.78%) occurred in 2013 and 73 (80.22%) in 2014. There were 76 outbreaks (87.51%) occurred in three provinces: 45 (51.14%) in Osmaniye, 23 (26.14%) in Adana and 8 (10.23%) in Kahramanmaraş. There were outbreaks on 624 of 187.199 farms (0.33%) in all nine provinces, in which there are a total of 1.269.976 susceptible animals. In farms with outbreaks, 860 of 131.708 susceptible cattle had the disease (Prevalence Rate: 0.65%) and 249 cattle died (Mortality Rate: 0.19%) (Türkvet, 2014). Outbreak reports showed that there was a decrease in disease transmission in 2014, probably as a result of successful disease control precautions, quarantines, and vaccinations carried out in 2013. There is still a risk of the disease spreading to other provinces. The mortality, prevalence, and fatality rates observed in 2013 were higher than in 2014.

Osmaniye province had the highest rate of infected farms (1.87%) followed by Adana province (0.83%). The animal-level prevalence rates were the highest in Hakkari (28.57%) and Batman provinces (18.75%), lower in Osmaniye (0.3%) and Malatya (0.25%), and the lowest in Sivas province (0.14%).

**Table 2.** Values with multivariable logistic regression for investigated risk factors for LSD in Turkey between 2013 and 2014.

| Factors | | S.E. | Wald-value | Odds Ratio | **95% CI** | ***P*- value** |
|---|---|---|---|---|---|---|
| **Age*** | 3.049 | 0.444 | 47.07 | 21.10 | 8.83-50.43 | 0.001 |
| **Sex*** | 2,960 | 1,051 | 7.94 | 19.29 | 2,46-151,32 | 0.005 |
| **Vaccination*** | -2.030 | 0.480 | 17.90 | 0.13 | 0.05-0.34 | 0.001 |
| **Constant** | -0.087 | 0.417 | 1.71 | 0.91 | | |

*ref:<24 month; female, vaccinated

Regarding animal movement from infected provinces to non-infected ones, it was observed that Kayseri, Düzce, Kırşehir, İzmir, Konya, and Gaziantep provinces are under risk of transmission. Kayseri borders three infected provinces (Sivas, Kahramanmaraş, and Adana), and Gaziantep borders four infected provinces (Adıyaman, Kahramanmaraş, Osmaniye, and Hatay) (Türkvet, 2014).

In this study, a GIS-based system was created by the integration of all data. Geographical queries and analysis were performed by user interface programs developed using ArcGIS 10.1 GIS software (Fig. 2). The risk assessment in different periods and queries were implemented by pressing one button after entering the necessary parameters. Additionally, thematic maps such as prevalence, case fatality, and mortality rates were produced for facilitating interpretations about animal health by the GIS-based system (Fig. 3).

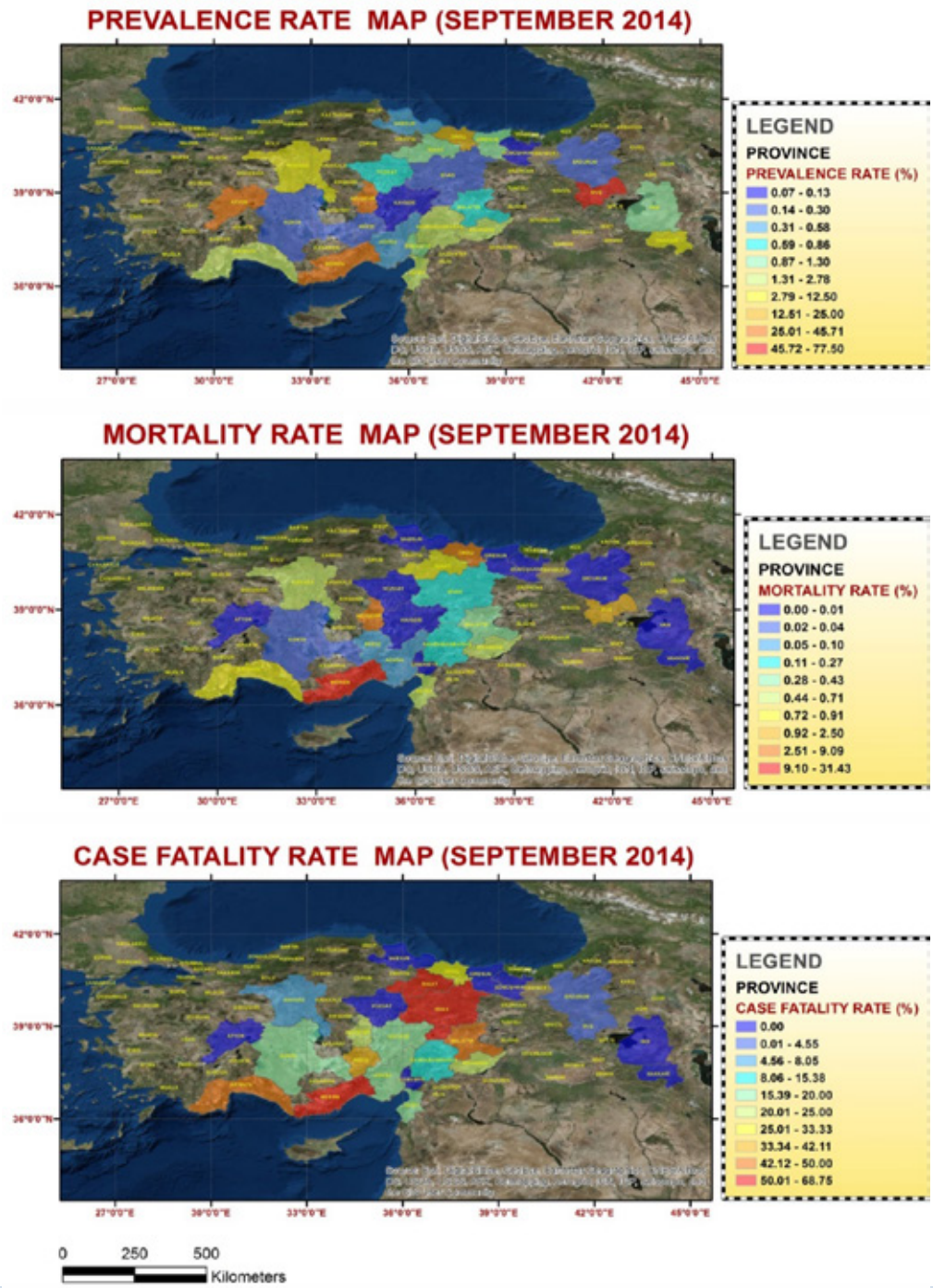


**Fig 3** Prevalence Rate (PR), Mortality Rate (MR) and Case Fatality Rate (CFR) maps produced by GIS platform.

## DISCUSSION

LSD prevalence and its association with different risk factors were analyzed. Mixed and local breeds of cattle were examined in terms of variable morbidity and fatality rates. Mixed and local breeds had almost the same rate, 26.04% and 38.18%, respectively. LSD morbidity ranged from 3% to 85%, and mortality was limited to 3% (Babiuk et al., 2008, Tuppurainen and Oura, 2012, Alemayehu et al., 2013).

The mortality and fatality rates were 2.56% and 9.85%, respectively, which showed that mixed breed was more resistant than local breed against the disease. In the present study, an attempt was made to compare the susceptibility of the mixed and local breeds of cattle raised in the same farming system. Analysis of the association among age, sex and vaccination status of animals sick with LSD revealed statistically significant results. This agrees with previously conducted studies on breed-specific LSD tendencies (Woods, 1988, Davies, 1991, Ayelet et al., 2013).

Vaccinations with the SP(Bk) sheep pox vaccine strain were used to control LSD. There were various reasons for failure of vaccination such as a low titer of the vaccine, different field and vaccination strains, vaccination of calves with maternal antibodies, and mishandling of vaccines in transport or storage. In reports from Israel (Brenner et al., 2006) and Egypt (Fayez and Ahmed, 2011), results are similar.

In terms of age, morbidity was higher in 24-month-old cattle, 24-48-month cattle, and cattle older than 48 months in mixed breeds. The results agree with those from Israel (Fayez and Ahmed, 2011) and Egypt (Ayelet et al., 2013). Morbidity is higher in females because the lactation or pregnancy period causes physiological stress and lowers immunity. Another attempt was made in the present study to compare the season during which an outbreak of the disease can occur. It was high in the summer season (45.45%) and the lowest in the spring season (13.33%) in the area. LSD outbreaks occurred during dry seasons and were more common while traveling at long distances (Brenner et al., 2006, Kumar, 2011, Magori-Cohen et al., 2012), similarly to the 2014 outbreaks in Turkey. LSD appeared in Egypt and then spread to the Middle East and Israel (Tuppurainen and Oura, 2012) as well as Kuwait, Lebanon, the UAE, Israel, and Oman in 1990. The occurrence of the disease depends on factors such as animal movements, immune status, wind, and the amount of rainfall (Brenner et al., 2006). According to the OIE reports, legal or illegal animal movements in Azerbaijan, Iran, Lebanon, Egypt, and Palestine are likely to have caused the virus to spread after the 2014 outbreaks (EFSA, 2015).

Analyses of morbidity risk factors showed that cattle purchased from other farms are at risk. For the transmission of LSD among farms, the most significant factor was cattle movement. The transmission of the disease to Turkey may be from Syria and Iraq since there is a movement of live animals across the Syria–Iraq border. Furthermore, the first outbreak near the border enables airborne vectors. According to the study, LSD prevalence was significantly associated with purchasing infected animals that were not tested or quarantined. This situation is similar to the situation in Ethiopia (Gari et al., 2010, Gari et al., 2011, Gari et al., 2012) and Egypt (Salib and Osman, 2011).

It is likely that live animal movements and smuggling occur across the south-eastern border with Syria and Iraq. This study's results agree with those from Jordan (Abutarbush et al., 2013).

The results of the present study are consistent with those that have been previously reported. LSD occurs based on factors such as the prevalence of insect vectors, number of livestock and transportation of animals that have not been tested (Ali et al., 1990, Tuppurainen and Oura, 2012). It is more likely to occur via animal movements than through arthropod vectors (Tuppurainen and Oura, 2014).

Hence, the main factor in LSD distribution is animal and insect movement. Importing infected animals is the main cause of LSD, so the disease has begun to spread over long distances. Further spread can be prevented by stopping the movement of sick animals to unaffected areas (Işıdan et al.,2014).

According to the 2014 reports (Işıdan et al., 2014), disease outbreaks in Sivas province, 400 km north of the initial outbreak, supported the possibility that LSD spread depending on arthropod vectors and illegal animal movements. The illegal movement of sick or asymptomatic infected animals was the possible cause (EFSA, 2015).

## CONCLUSIONS

In conclusion, control strategies for LSD infection on farms should focus on these risk factors. These risk factors could relapse the spread of LSD, and it could be found spreading to new regions that have been pre-

viously considered as a free region and will be a major livestock breeding health problem. The findings of this study also attach attention to the distribution of LSD in the area and can assist decision-makers, planners, and researchers in their efforts. Briefly, all queries, spatial analysis, and documentation processes can be performed on the GIS-based system developed. Thus, risk assessments and evaluations about animal health can be performed easily and effectively by GIS.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Influence of dietary *Moringa oleifera* on broilers performance, intestinal microbial population and humoral immune competence

**W. A. Abd El-Ghany*[1], R. D. Eraky[2]**

[1]*Cairo University, Faculty of Veterinary Medicine, Department of Poultry Diseases, Giza, Egypt*

[2]*Animal Health Research Institute (Damietta branch), [2]Bacteriology Department, Damietta, Egypt*

**ABSTRACT:** This study was designed to evaluate the effect of using different levels of phytobiotic containing *Moringa oleifera* (*M. oleifera*) leaf powder (MOLP) on broiler chickens body growth performance parameters, intestinal microbial population, and the humoral immune response. Day-old Hubbard broiler chicks ($n$ = 200) were randomly allocated into 4 treatment diet. Basal diet was supplied with treatments T0, T1, T2 and T3 representing (0, 1%, 5% and 7.5% MOLP); respectively. Chickens were kept under observation for 5 weeks. Body performance parameters, total viable bacterial and coliform counts and humoral immune response to Newcastle disease (ND) virus vaccine were detected using the haemagglutination inhibition (HI) test. The results revealed significant ($P < 0.05$) improvement in performance parameters in groups supplemented with dietary MOLP. However, the best significant ($P < 0.05$) performance was observed in the group fed 1% MOLP. There was no significant ($P < 0.05$) difference among treatments considering the total viable intestinal bacterial and coliform counts. Significant ($P < 0.05$) increase in the means of HI in dietary MOLP supplemented groups was observed, where the highest means were seen in 1% MOLP treated birds. In conclusion, dietary supplementation with MOLP could improve the performance parameters and the immune response while reducing the total viable intestinal bacterial and coliform counts of broiler chickens. The study recommended using of dietary level of 1% MOLP to improve performance, intestinal health, and immune competence.

***Keywords:*** *M. oleifera*, chickens, growth, bacterial count, HI

*Corresponding Author:*
Wafaa Abd El-Ghany Abd El-Ghany, Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, Egypt, Giza Square, 12211
E-mail address: wafaa.ghany@yahoo.com

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## INTRODUCTION

Antibiotic growth promoters have been widely used for the prevention of poultry infection and improvement of meat and egg production. Using antibiotics as growth promoters have been banned in several countries due to the development of bacterial drug resistance, presence of drug tissue residues and destruction of normal gut microflora (Hong et al., 2012; Kostadinović et al., 2019). Nowadays, supplementation of alternatives phytogenic feed additives in poultry ration is commonly anticipated as growth promoters to enhance the performance of broiler chickens (Hashemipour et al., 2016; Popović et al., 2018).

Leaf powder of *M. oleifera* is considered as chep source of poultry ration protein (Tesfaye et al., 2013), in addition to vitamins, acids, minerals, and various phenolics compounds (Moyo et al., 2012). Dietary supplementation of poultry with *M. oleifera* leaf powder (MOLP) improved the body performance traits (Okafor et al., 2014; Mousa et al., 2017), as well as enhanced both the intestinal health (Gomashe et al., 2014) and the humoral immune response (Eze et al., 2013 and 2014).

Therefore, this work was planned to detect the influence of using different levels of MOLP on body growth performance parameters, intestinal microbial population and humoral immune response of broiler chickens.

## MATERIALS AND METHODS

### Experimental chickens and design

Day-old Hubbard broiler chicks were obtained from a commercial hatchery. Birds were fed on starter, grower and finisher diets at ages 2, 2-4 and 4-5 weeks, respectively. No antibiotics or coccidiostats was added to the ration. The ration was formulated to meet the nutrient requirements of broiler chicks according to NRC (1994) which were formulated from the local feed ingredients commonly used for poultry feed in Egypt. The main ingredients composition of diets is represented in Table 1. Feed and water were given *ad libitum*. Vaccination was done against Newcastle disease (ND) and infectious bronchitis viruses using living Hitchner HB1 and H120 strains, respectively at 5 days old, against avian influenza (AI) virus using inactivated H5N1 strain at 7 days old, against Gumboro disease virus using intermediate strain at 12 days old and against ND virus using La Sota strain at 19 days old. Vaccines were administrated using eye drop method except AI vaccine using a subcutaneous inoculation method. A total of two hundred, one day old mixed broiler chicks were randomly allocated into 4 equal groups of 50 chicks. Each group was further subdivided into 2 replicates with 25 chicks per each. The chicks of each replicate were housed in thoroughly cleaned and disinfected deep litter houses. Basal diet was supplied with different levels of MOLP. Treatments were T0, T1, T2, and T3 representing 0, 1%, 5% and 7.5% MOLP, respectively they were fed for 5 weeks of experimental study. The study was done in accordance with the National regulations on animal welfare and Institutional Animal Ethical Committee recommendations and approval.

### *M. oleifera* plant

The leaf of *M. oleifera* was obtained from the Agricultural Research Center, Giza, Egypt. They are dried and ground as a powder. The powder of MOLP was subjected to proximate analysis according to the Association of Official Analytical Chemists (AOAC, 2000) methods. The composition of plant leaves extract was the followings; 26.2% crude protein, 18.0% crude fiber, 3.0% crude fat, 6.0% ash and 10.0% moisture content. It was added to the ration in different concentrations (0, 1%, 5% and 7.5%).

### Studied parameters

#### Performance variables

At day old arrival, all birds were weighed and then weekly weighed till the end of the experiment (5 weeks old). The feed consumption was also recorded weekly (g feed/g body weight). The FCR was estimated by dividing the total feed consumption/Kg by the total body weights/Kg. The performance parameters variables were estimated according to Bell and Weaver (2002). The birds were observed daily for signs or mortalities. The European production efficiency factor (EPEF) for each group was calculated at the end of the study as the following; [Average live body weight (Kg) × Liveability (%)] × 100 / [Marketing age (day) × FCR].

#### Intestinal bacterial count

At the end of the study (5 weeks old), ten birds from each treatment were sacrificed and the intestinal contents were collected for determination of total viable bacterial count and coliform count (ISO, 1995). The results of the total bacterial count were expressed as the number of the organism of colony-forming units per gram (CFU/g) contents of the intestine.

**Humoral immune response**

Blood samples were collected weekly from the wing vein of ten birds in each treatment. The serum was separated by blood centrifugation. The blood was kept in refrigerator for several hours, and then centrifuged for serum. The titers of humoral immune response were investigated using the haemagglutination inhibition (HI) test with 8 haemagglutinating units (Swayne et al., 1998).

**Statistical analysis**

The results were statistically analyzed using the General Linear Model procedure of the Statistical Analysis System software (SAS®, 2000). Overall data were analyzed using one way ANOVA test.

Significant differences between treatment means were considered significant at level P <0.05.

**Table 1.** Main ingredients composition of diets given for broiler chickens for 5 weeks

| Elements (g/kg feed) | Type of diets | | |
|---|---|---|---|
| | Starter | Grower | Finisher |
| Metabolized energy (kcal/kg) | 3000 | 3150 | 3200 |
| Crude protein % | 23.0 | 22.0 | 19.0 |
| Soybean meal (45%) | 330.5 | 302.7 | 250.9 |
| Yellow maize (9%) | 57.94 | 57.94 | 57.94 |
| Maize gluten meal (60%) | 70.2 | 70.1 | 65.7 |
| Fat | 32 | 45 | 41 |
| Lysine | 2.9 | 2.7 | 3.5 |
| Methionine | 2.2 | 2.0 | 2.2 |
| Dicalcium phosphate | 18 | 18 | 18 |
| Sodium chloride | 4 | 4 | 4 |

Each g of the mineral mixture of the diet contained: IU: vit. A 9000, vit. D3 2500, vit. E 17; mg, vit. K3 2.5, vit. B1 1.7, vit. B2 6.6, vit. B6 2.4, vit. B12 0.015; mg choline chloride 400, Mn 80, Fe 40, Zn 70, Cu 8, Se 0.3.

## RESULTS AND DISCUSSION

There was no clinical signs or mortalities was recorded during the experimental period.

The results of Table 2, showed significant (P < 0.05) improvement in broilers body weights with different levels of MOLP (1, 5 and 7.5%). Similarly, Nkukwana (2012) found that birds supplemented with MOLP had higher body weight than the birds fed the control diets throughout the production period. Treatment fed on a diet with 1% *M. oleifrea* leaf meal gained significantly (P < 0.05) higher body weights than the control group and other treatments with higher levels (5 and 7.5%). Lower concentrations of MOLP were found to induce a positive significant effect on broilers performance than a higher one. This observation is supported by the study of Olugbemi et al. (2010) found that increasing the inclusion level of *M. oleifera* leaf meals in broiler diets results in depressed growth performance. Khan et al. (2017) observed that among the levels of MOLP included in the basal diets (i.e., 0.6, 0.9, 1.2 and 1.5%), only MOLP at the level of 1.2% that increased the body weight gain, whereas the other levels did not exert any effect. Kout et al. (2015) and Agashe et al. (2017) observed significantly higher body weights on diets containing different levels (0.2, 0.4 and 0.6%) of *M. oleifera* leaf meal. Divya et al. (2014) demonstrated that addition of MOLP at 0.5, 1.0, 1.5 and 2.0% levels or antibiotic slightly decreased body weight. Ochi et al. (2015) also found significant reduction in weight gain and body weight at inclusion level of 2% *M. oleifera*, while supplementation with 0.5% resulted in significant increase in feed consumption. Authors have speculated that this may be due to the presence of phytate which acted as an anti-nutritional factor. Bitter test of *M. oleifera* leaves (tannin at 1-23 g/kg) inducing reduced palatability and feed intake of birds when consumed in higher levels (Onunkwo and George, 2015).

Dietary treatments with *M. oleifera* showed better FCR when compared with control treatment. The best FCR was obtained by using 1% MOLP in all periods compared to other higher treatment levels and control (Table 3). These results are in agreement with Banjo (2012) who found that inclusion of *M. oleifera* leaf meal at 1, 2 and 3% in the diet did not significantly enhance FCR. Onunkwo and George (2015) reported a significant decrease in FCR of the birds fed MOLP levels 0.0, 5.0, 7.5 and 10%. Inoculation of low levels of MOLP was investigated by Aderinola et al. (2013) who recorded better FCR after feeding of broilers

with *M. oleifera* leaves meal-based diets (0, 0.5, 1.0, 1.5 and 2.0%) than control. Also, Kout et al. (2015) recorded the best FCR in birds fed on 0.2% MOLP.

The results showed no significant (P < 0.05) difference among treated groups and the control one (Table 4). The improvement in the performance of broilers observed due to the supplementation of MOLP may also be attributed to the significant quantities of vitamins (A, B and C), calcium, iron, and protein. The leaves of *M. oleifera* have been reported to have an antioxidant activity due to the higher amount of polyphenols, tannins, anthocyanin, glycosides, and thiocarbamates, which remove free radicals, activate antioxidant enzymes and inhibit oxidases (Moyo et al., 2012).

Table 5 reveals that there was no significant (P > 0.05) difference in the means of mean total viable intestinal bacterial and coliform count among different treatments. Nevertheless, 1% MOLP revealed the lowest total vial intestinal bacterial count as compared with control. Parallel results were seen by Moez et al. (2014) who detected enhancement of the antimicrobial activity of dry *M. oleifera* leaves. Divya et al. (2014) detected that supplementation of MOLP (0.5, 1.0, 1.5 and 2.0%) significantly reduced the gut microflora and coliform population. Moreover, Onsare et al. (2013), Gomashe et al. (2014) and Hossam et al. (2016) observed that *M. oleifera* leaf extract inhibited the growth of *Escherichia coli* and *Salmonella* spp. and act as antibacterial. Yang et al. (2006) and Younis and Elbestawy (2017) proved the positive effect of *M. oleifera* (3% dried leaves) on the enhancement of duodenum traits, reduced *E. coli* and increased *Lactobacillus* spp. counts which helped in improving the intestinal health of broilers and increasing the production of digestive secretions and nutrient absorption. Al-Husnan and Alkahtani (2016) demonstrated that MOLP possesses antibacterial properties that may be beneficial in lowering the populations of potentially pathogenic bacteria in broilers intestine. Extracts of *M. oleifera* seeds may contain antibiotic metabolites, such as carboxylic acid, 2,4-diacetyl phloroglucinol, and cell wall-degrading enzymes and chitinases. It also possesses antimicrobial activity due to its principle component pterygospermin which kills bacteria by blocking the enzymes cysteine proteinase and alcohol dehydrogenase and thus blocks bacteria metabolism and survival (Yang et al., 2006).

Our findings showed that there were significant (P < 0.05) differences in the means of HI titers between MOLP and the control where the highest means were seen in 1% MOLP treated birds (Table 6). Several investigations are indicative of beneficial effects of *M. oleifera* in boosting the immune systems (Olugbemi et al., 2010;Al-Majali et al., 2017). The study of Yang et al. (2006) showed that dietary dehydrated leaves of Moringa induce a significant increase in the concentrations of total globulin, γ-globulin and lymphocyte ratio, antibody titer levels with comparison to the standard (sheep erythrocytes), and delayed-type hypersensitivity of broiler chickens. Comparable results also were obtained by Chollom et al. (2012), Eze et al. (2013), Hossam et al. (2016) and Younis and Elbestawy (2017) who demonstrated that *M. oleifera* extracts have nutritional value as well as strong antiviral activity against ND virus.

**Table 2.** The effect of different treatments level of MOLP on body weights of broiler chickens

| Treatments | Age in weeks | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 |
| T0 | 46.61±0.7 | 86.10±1.6$^{b}$ | 253.31±3.0$^{c}$ | 675.01 ±6.9$^{c}$ | 970.80±12.1$^{c}$ | 1296.19±16.9$^{c}$ |
| T1 | 45.82±0.6 | 109.40±1.4$^{a}$ | 310.65±5.0$^{a}$ | 786.76±5.7$^{a}$ | 1290.72±11.7$^{a}$ | 1907.45±14.3$^{a}$ |
| T2 | 44.91±0.3 | 102.75±1.1$^{a}$ | 296.93±4.6$^{b}$ | 754.84±5.9$^{b}$ | 1198.52±11.9$^{a}$ | 1710.21±14.6$^{b}$ |
| T3 | 45.35±0.5 | 107.98±1.5$^{a}$ | 291.87±4.0$^{b}$ | 715.93±5.1$^{ab}$ | 1065.43±12.0$^{b}$ | 1800.76±14.9$^{b}$ |

Means with different superscripts, within age, are significantly different (P < 0.05)

**Table 3. The effect of different treatments level of MOLP on FCR of broiler chickens**

| Treatments | Age in weeks | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| T0 | 1.50±0.20 | 3.10±0.41$^{a}$ | 2.97± 0.31$^{a}$ | 2.76 ± 0.72$^{a}$ | 3.09 ± 0.21$^{a}$ |
| T1 | 1.21±0.03 | 1.56±0.05$^{c}$ | 1.42±0.02$^{c}$ | 1.63±0.04$^{c}$ | 1.27±0.03$^{c}$ |
| T2 | 1.35±0.02 | 1.90±0.03$^{b}$ | 1.69±0.01$^{ab}$ | 1.87±0.03$^{b}$ | 1.63±0.02$^{b}$ |
| T3 | 1.38±0.01 | 2.09±0.02$^{b}$ | 1.90±0.01$^{b}$ | 1.94±0.02$^{b}$ | 1.67±0.02$^{b}$ |

Means with different superscripts are significantly different (P < 0.05)

**Table 4.** The effect of different treatments level of MOLP on EPEF of broiler chickens

| Treatments | European Production Efficiency Factor (EPEF) |
|---|---|
| T0 | 275.5±7.91 |
| T1 | 298.3±6.84 |
| T2 | 283.7±8.0 |
| T3 | 279.9±9.76 |

**Table 5.** The effect of different treatments level of MOLP on total viable count and Coliform count in broiler chickens

| Treatment | Total viable count log10 (CFU/g) | Coliform count log10 (CFU/g) |
|---|---|---|
| T0 | 6.2 | 5.8 |
| T1 | 5.7 | 4.9 |
| T2 | 5.9 | 5.2 |
| T3 | 6.1 | 5.6 |

**Table 6.** The effect of different treatments level of MOLP on HI antibody titers against ND virus vaccine in broiler chickens

| | Age in weeks | | | | |
|---|---|---|---|---|---|
| Treatments | 1 | 2 | 3 | 4 | 5 |
| T0 | 4.7 ±3.0[a] | 3.9±3.5[a] | 4.8±3.0[a] | 5.1±3.2[a] | 5.03±2.2[a] |
| T1 | 5.9±2.5[b] | 4.7±2.9[b] | 6.5±2.1[b] | 6.3±2.8[b] | 6.8±1.7[b] |
| T2 | 5.6±2.3[b] | 4.7±2.9[b] | 6.3±3.4[b] | 6.0±2.8[b] | 6.5±1.7[b] |
| T3 | 5.4±2.3[b] | 4.2±3.7[b] | 6.1±3.4[b] | 6.1±3.0[b] | 6.2±1.8[b] |

Means with different superscripts are significantly different ($P < 0.05$)

## CONCLUSIONS

In conclusion, dietary supplementation of broiler chickens with levels 0, 1%, 5% and 7.5% MOLP could improve the body weight, FCR and EPEF, enhance the gut health by reducing the total and coliform bacterial count as well as boost the titers of HI humeral immune response to ND virus vaccine. However, 1% of MOLP induced the best results among the other levels. So, it is recommended to include this level in broiler ration.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

# Investigation of serum homocysteine levels in relation to age and sex

**N. H. Aksoy**

*Department of Biochemistry, Faculty of Veterinary Medicine, Aksaray University, 68100 Aksaray, Turkey*

**ABSTRACT.** Homocysteine is a non-proteinogenic and a derived amino acid in the methionine metabolism and is a risk factor for cardiovascular and many other metabolic diseases. In this study, the purpose was to determine serum homocysteine levels in healthy sheep based on differences in age and gender. 220 healthy Akkaraman sheep, composed of both females (n=55 lambs and 55 ewes) and males (n=55 lambs and 55 rams), were used as animal samples. The measurements of serum homocysteine concentrations were performed with ELISA-HCY kit. The levels of serum homocysteine of sheep were detected in ewes, female lambs, rams and male lambs as 2,91±0,50; 2,99±0,42; 11,22±3,10; 6,43±1,26 µmol/L, respectively. The primary intent of this study was to investigation and characterization the serum Hcy concentrations in healthy sheep broken down by different ages in both genders As a result, the serum homocysteine values that can constitute a reference value for healthy breeds of sheep were determined in this study.

***Keywords:*** *Sheep, homocysteine, gender differences, age differences*

*Corresponding Author:*
N. H. Aksoy, Department of Biochemistry, Faculty of Veterinary Medicine, Aksaray University, 68100 Aksaray, Turkey
E-mail address: nhaksoy@gmail.com

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## INTRODUCTION

Homocysteine (Hcy), a sulphur-including amino acid that does not participate in protein construction and DNA structure, was first isolated from urine bag by Vincent du Vigneaud in 1933 and in 1955, for which he received the Nobel Prize in Chemistry (McCully 1969; Wijekoon et al., 2006; Graham et al., 1997; Ramakrishnan et al., 2006).

For the first time, McCully (1969) proposed a "homocysteine theory in atherosclerosis" and has developed the hypothesis that homocysteine may lead to vascular diseases (McCully 1969; Graham et al., 1997; Wijekoon et al., 2006).

Hcy is an intermediary product in methionine metabolism. Hcy, which mediates methionine metabolism, is catabolized in the reaction with the vitamin B6 cofactor of Cysteine (Cys). In most tissues, Hcy can be converted back to methionine by this re-methylation reaction, catalyzed by the enzyme "methionine synthase," requiring 5-methylene tetrahydrofolate (THF4) in the form of reduced folate as the methyl donor with vitamin B12 cofactor (Finkelstein et al., 2000; Ravaglia et al., 2005; Wijekoon et al., 2006; Ramakrishnan et al., 2006; Martinez et al., 2017; Mohammad et al., 2010; Kumar et al., 2017; Škovierová et al., 2016).

The Hcy formed inside the cell is released into the blood and urine. Vitamin B12, folate and B6 are essential cofactors in Hcy re-methylation and trans-sulfation pathways. Plasma total Hcy levels are known and accepted to be important biochemical parameters as determinants of possible cardiovascular conditions and consequent mortality in patients with neuronal disorders, renal insufficiency, diabetes, systemic lupus erythematosus and venous thromboembolism, especially coronary-heart disease (Martinez et al., 2017; Ramakrishnan et al., 2006; Kumar et al., 2017). The conclusions of different studies clearly showed that insufficiencies of folate, vitamin B12, or methionin are affecting serum and cellular levels of S-adenosylmethionine (SAM), S-adenosylhomocysteine and homocysteine and are linked with molecular processes leading to oncogenesis and also carcinogenesis (Murin et al., 2017; Martinez et al., 2017).

Human studies have shown that plasma Hcy concentrations increase with age (higher in adults than in pediatric periods), and are higher in males than females. It is known that the most important factors affecting Hcy level are genetic and nutritional factors (vitamin B6 and B12 or folate deficiencies), age, gender difference, physiological periods, especially the postmenopausal period and some metabolic disorders (Robinson et al., 1994; Mohammad et al., 2010; Piccione et al., 2008; Masuda et al., 2008; Škovierová et al., 2016).

While slight age-related increases are considered physiological, the high Hcy levels observed at younger ages are linked to genetic predisposition and environmental factors (smoking, toxic carcinogenic substances and drugs) (Corrales et al., 2002; Carmel and Jacobsen, 2001; Refsum 1998; Wijekoon et al., 2006; Varga 2005; Richards 2008; Hankey and Eikelboom 1999; Graham et al., 1997; Martinez et al. 2017; Ramakrishnan et al., 2006; Piccione et al., 2008). An increase in the homocysteine level is an indicator of lower intake of foods containing vitamin B12, or malfunction of renal functions or possibly a low enzyme activity in the metabolism of homocysteine (Martinez et al., 2017; Furlong et al., 2010; Murin et al., 2017).

With this current preliminary research, is aimed that, to reveal and compare the presence of homocysteine in sheep, depending on age and gender differences.

## MATERIALS AND METHODS

### Ethical scope

This study was conducted in accordance with the principles of the Local Ethics Committee in the framework of the ethics confirmed by the Bahri Dagdas International Agricultural Research Institute, Directorate of Local Ethics Committee of Animal Experiments (**14.01.2015 / 35 and 0088).**

### Sample collection

The sample collection was done during May 2016. The sheep were on pasture free-ranging and normal-fed, and the ambient temperature was 1.5 °C above the normal seasonal average for May. In this study, blood samples were collected from animals in private enterprises located in Aksaray, Turkey. The animal material in this study consisted of 220 healthy Akkaraman sheep. The sheep originated from Aksaray and its vicinity. All animals were found healthy by clinical examination and general clinical perspective. The animals were separated into four groups of 55 sheep each, based on sex and age. Two hundred and twenty totally healthy sheep, both females (n=55 lambs and 55 ewes) and males (n=55 lambs and 55 rams), were used as animal material.

Blood samples were simultaneously collected during morning hours from each animal. 15 mL of animal blood was taken from the jugular vein and placed into ice-bed-cold vacuum serum-separating tubes as appropriate for the blood-collection procedure. Blood samples, which were collected into anticoagulant-free serum tubes, were centrifuged 10 minutes at 3000 rpm (Coles, 1986) in biochemistry laboratory. Hemolysis-free sera obtained after centrifugation were separated into the micro (Eppendorf) tubes and labeled and stored at -25 °C deep freeze until analyzed. These samples were used for homocysteine analysis.

**Hcy assays**

For the Hcy assay, previously frozen serum samples were dissolved (at RT) and assayed at without wasting time. They were analyzed in the same experimental set and against the blind.

Levels of serum peptides were measured blindly by using commercially available enzyme-linked immunosorbent assay (ELISA) kits to detect peptides in the biological fluids and were read with using the ELISA plate reader.

In the scope of the study quantities of Hcy peptides in sera were studied by commercial quantification (Shangai Sunred, Biological Tech., China) using the ELISA method. HCY-ELISA-Plates were read on the ELISA-Plate Reader (450 nm) (ELx800 Absorbance Microplate Reader-Biotek). Homocysteine concentrations were calculated from standard curves.

**Assay range:** 0.6µmol/L→100µmol/L.

**Sensitivity:**0.31µmol/L.

CV (%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

**Statistical analysis**

Descriptive statistics for the properties studied; Mean, Standard Deviation, Standard Error, Minimum and Maximum values.

Data were analyzed using the SPSS 15.0 for WindowsTM statistical software (SPSS Inc., Chicago, IL, USA). Differences among the groups were analyzed by Student t-test. "One-way ANOVA" was performed to compare the group averages in terms of continuous variables. The Duncan multiple comparison test was used to identify the different groups following the analysis of variance. The data are given as the means ± standard error (X ± SH). Statistical significance was accepted as $p<0.05$ level.

**RESULTS**

The serum Hcy levels of the four groups, which differs based on age and sex, were presented in table 1. In the current study, serum homocysteine levels (table 1) were found to be higher in males than in females when examined without regard to age (figure 3, 4). It was found that in females, age difference in Hcy values was not found statistically significant (Hcy values were found to be close to each other in female lambs and ewes, figure 1). In males, it was much lower in lambs than in rams (figure 2). These differences were significant compared to rams ($p<0.05$) (table 1).

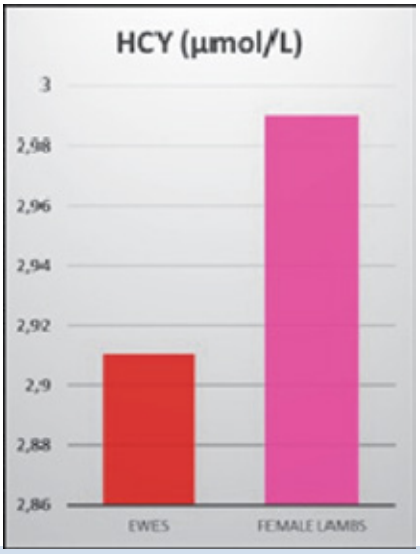


**Fig. 1.** Levels of serum Hcy in ewes and female lambs

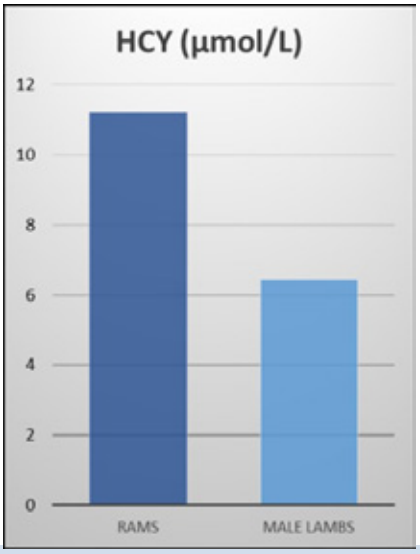


**Fig. 2.** Levels of serum Hcy in rams and male lambs

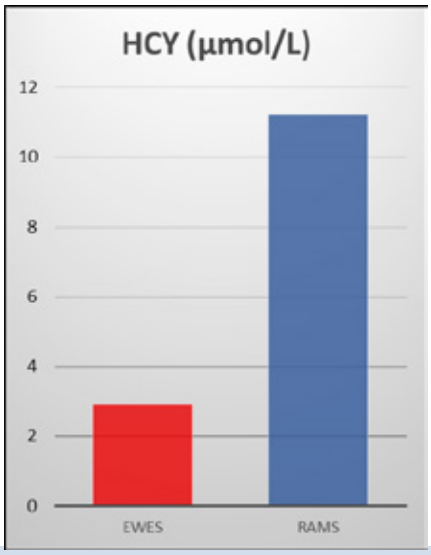


**Fig. 3.** Comparing the serum Hcy levels of ewes and rams

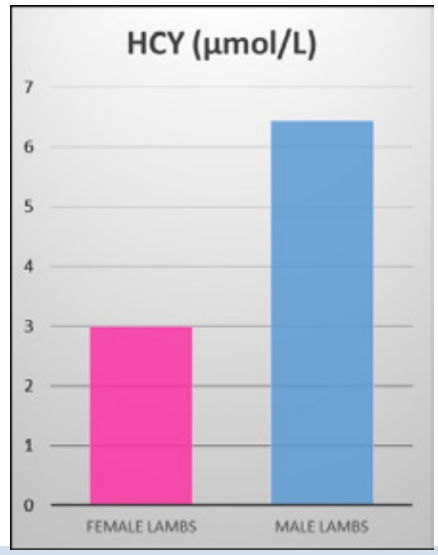


**Fig. 4.** Comparing the serum Hcy levels of female and male lambs

**Table 1.** Levels of serum Homocysteine of Akkaraman sheep

| Polypeptide (Unit) | Ewes (n=55) | Female lambs (n=55) | Rams (n=55) | Male lambs (n=55) |
|---|---|---|---|---|
| Homocysteine (µmol/L) | 2,91±0,50a | 2,99±0,42a | 11,22±3,10ab | 6,43±1,26ab |

## DISCUSSION

From 1969 until today, a variety of research was performed based on different viewpoints on Hcy. These first studies have been carried out in especially humans (Carson et al., 1963; Gibson et al., 1964; McCully 1969; Graham et al., 1997).

Hcy is known to be an important biochemical parameter, primarily as a cardiovascular marker, as a determinant of neuronal disorders, renal health, renal failure, diabetes and venous thromboembolism, and even carcinogenesis (Finkelstein et al., 2000; Ravaglia et al., 2005; Wijekoon et al., 2006; Martinez et al., 2017; Ramakrishnan et al., 2006; Kumar et al., 2017). Levels of the advanced biochemical research marker Hcy are a current and reliable tool for the evaluation of the health conditions of humans and animals in different metabolic and endocrinal periods in which the homeostatic continuum of organisms is altered. The analysis of Hcy amounts is a valuable parameter for the assessment of the health status of an animal in different endocrinal periods such as the pre-menopausal, post-menopausal or lactation period, etc (Piccione et al., 2008). Also in terms of homocysteine, gene, breed, age and sex differences influences the biochemical, physiological, endocrinal and all metabolic variables of organisms. Therefore, it is important to consider the homocysteine levels as a trustworthy marker of human and animal welfare in order to demonstrate possible preventive changes of the healthy states and the beginning of the diseases and metabolic disorders.

The main goal of this research was to analysis and qualifies the serum Hcy concentrations in healthy sheep broken down by different ages and genders. It could not found many research reference amounts for small ruminants (especially for sheep) on this subject. The current research was performed to find out normal physiological values of serum Hcy concentrations in the healthy Akkaraman breed sheep.

In human studies have shown that concentrations of plasma Hcy increase with age and are higher in males than females. This result shows that homocysteine levels are determined by both genetic and nutritional factors and gender differences are important (Robinson et al., 1994; Graham et al., 1997; Hankey and Eikelboom 1999; Corrales et al., 2002; Varga 2005; Wijekoon et al 2006; Richards 2008; Piccione et al., 2008; Masuda et al., 2008; Moham-

mad et al., 2010; Škovierová et al., 2016).

In the present study, results were obtained in a similar framework. Compared to the submitted work, homocysteine levels were found to be higher in males than females without regard to age. Levels were much lower in male lambs than in rams and it was observed that these results were statistically significant compared to rams ($p<0.05$). It was detected that for Hcy values, age differences were not very important in females (levels were found to be close to each other in female lambs and ewes).

In the literature, related research on the topic is sparse.

A study conducted by Furlong and colleagues in 2010 aimed to assess the possible diagnostic role of plasma Hcy in relation to methylmalonic acid and vit B12, in pregnant Romney ewes (3 years). They reported the mean concentrations of Hcy remained within the range of 1.5–3.0 µmol/L (Furlong et al., 2010). Its seen that, this result is very close to our Hcy levels in 1-4 years old ewes (2,91±0,50 µmol/L).

In other research Rezaei and Dalir-Naghadeh specified that, homocysteine amounts in lambs with the acute cardiac disease were higher than in lambs with the muscular dystrophy. Also they proved that Hcy concentrations in healthy lambs were lower than in with the cardiac form of acute selenium deficiency ones. The researchers declared that in sheep, increased plasma Hcy amounts would be a risk factor for myocardial disease (Rezaei and Dalir-Naghadeh, 2009). In another study, Kozat and coworkers had compared amounts of Hcy between healthy lambs and subclinical lambs with white muscle disease. They found that patient lambs have higher values of Hcy than healthy lambs ($p<0.05$). Compared with the present study (particularly male lambs, Hcy (6,43±1,26 µmol/L), the Hcy levels of healthy lambs (without sex discrimination) is very similar and values may be close (healthy lambs control group Hcy=5.10±3.33 µmol/L) (Kozat et al., 2011).

In a study managed by Piccione and his colleagues intended to investigate the effect of Hcy levels and the antioxidant stress of lactation in sheep, considering the lactation periods, only female sheep were selected (Piccione et al., 2008). In this study, serum Hcy was 2.91±0.50 (µmol/L) in female sheep (ewes) between 1-4 years old. It is observed that in Piccione's study, the level of serum Hcy determined on the first day of lactation was slightly higher (3.40±0.17 µmol /L) than the level in the current study (2.91±0.50 µmol/L). In a research study conducted in 2015, (Razavi et al., 2015), serum levels measured in relation to a parasitic infections (Malignant Ovine Theileriosis) in sheep were found to be lower in controls than in patient samples (control animal hcy level: 7.29±0.54; non-infected animal hcy level: 12.18±0.50; infected animal Hcy level: 11.16±0.57 µmol/L). In 2010, Mohammad and co-workers analyzed levels of serum homocysteine in male and female subjects below and above 50 years of age who have coronary heart disease with or without diabetes mellitus (Mohammad et al, 2010). Serum homocysteine levels were higher in patients with coronary heart disease and diabetes than in healthy controls. Further, serum homocysteine levels were lower in women than in men. This study additionally proved that the level of Hcy increases with age. But interestingly, in women over 50 years of age (post-menopausal period), the increase in Hcy level was slightly higher than in men.

## CONCLUSIONS

If we support our research with these and similar studies, measuring quantities of Hcy in plasma or serum as a metabolic indicator in sheep, especially the methylation capacity, and as a general health parameter will provide vital information on animal health.

Considering all conditions, differences in region, breeds, sex, age, season and nutritional sources could affect homocysteine levels and cause changes in the present study.

Consequently, it is intended that in presented study Hcy variables of age and gender of clinicaly healthy sheep were based on the reference values for healthy sheep. Amounts of blood Hcy get on the basis of breed, age and gender of sheep should meet the requirement for diagnosis of healthy conditions. This value might be a reference value for serum Hcy levels in healthy Akkaraman sheep.

The purpose of current work is to identfy and detect the serum Hcy values that are a biochemical, physiological, endocrinological, cardiological parameter and even carcinogenic biomarker of healthy sheep. These results will provide valuable contributions to the literature, for both academicians and veterinarians.

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## CONFLICTS OF INTEREST

The author has nothing to disclose. No conflicts of interest exist. The author has no financial or personal relationship that could inappropriately influence or bias the content of this paper.

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Research article
Ερευνητικό άρθρο

# Parasite infection in *Serranus cabrilla* (Perciformes, Serranidae): histopathological aspects and new host record for nematode genus *Philometra* from Aegean Sea, Turkey

**D. Innal[1*], M. M. Stavrescu-Bedivan[2], O. Ozmen[3]**

[1]*Burdur Mehmet Akif Ersoy University, Department of Biology, Burdur, Turkey*

[2] *University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Agriculture, Romania*

[3]*Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pathology, Burdur, Turkey*

**ABSTRACT.** This study was conducted to determine infection with nematode parasite *Philometra* sp. in gonads of *Serranus cabrilla* recorded in Izmir Gulf between October 2016 and July 2017. The overall prevalence was 14.46% and the mean intensity of infection 1.2 parasites per fish. The occurrence of philometrid infection on comber hosts was assessed according to several risk factors (fish length class, sex and season). Our findings suggest that the highest rates of parasite infection occur in larger sized fish, hermaphrodite individuals and during April month.

The present report also revealed that marked hyperemia was the major findings of infected gonads of *S. cabrilla*, where parasitic nematodes caused a marked inflammatory reaction at the histopathological examination.

As far as we know, this paper represents the first mention of genus *Philometra* in *S. cabrilla* from Turkey and the first presence of philometrid parasites in Aegean coast of Turkey. Furthermore, the present work is the first record of the effects of *Philometra* sp. parasitism on a serranid species in Izmir Gulf.

***Keywords:*** gonad, histopathology, infection, *Philometra*, *Serranus cabrilla*

*Corresponding Author:*
D. Innal, Burdur Mehmet Akif Ersoy University, Department of Biology, Burdur, Turkey
E-mail address: innald@yahoo.com

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## INTRODUCTION

The gonad-infecting parasites of *Philometra* (Nematoda: Phylometridae) occur in marine waters, causing harmful effects to their hosts (Perdikaris et al., 2003). These nematodes have a wide distribution in Atlantic, Indian and Pacific Oceans, damaging wild or cultured commercially important fish (Moravec et al., 2008). According to Moravec and de Buron (2013), the members of the Philometridae family are the most important group of dracunculoid nematodes parasitizing in the teleost fish.

The presence of four species of *Philometra* parasite genus has been already documented for helmintofauna of seven marine perciformes from Turkey: *P. filiformis* (Stossich, 1896) in Common pandora *Pagellus erythrinus* (Linnaeus, 1758); *P. globiceps* (Rudolphi, 1819) in Mediterranean horse mackerel *Trachurus mediterraneus* (Steindachner, 1868) and Stargazer *Uranoscopus scaber* (Linnaeus, 1758); *P. lateolabracis* (Yamaguti, 1935) in White grouper *Epinephelus aeneus* (Geoffroy Saint-Hilaire, 1817), Dusky grouper *Epinephelus marginatus* (Lowe, 1834) and Mottled grouper *Mycteroperca rubra* (Bloch, 1793); *P. saltatrix* (Ramachandran, 1973) in Bluefish *Pomatomus saltatrix* (Linnaeus, 1766); additionally, unnamed helminthes *Philometra* sp. were found in Goldblotch grouper *Epinephelus costae* (Steindachner, 1878) from Mediterranean Sea (Moravec and Genc, 2004; Öktener, 2014). Except the *P. globiceps* founded in the Black Sea, the other three knowing species of *Philometra* in Turkey were encountered in the Mediterranean Sea (Öktener, 2014).

The comber, *Serranus cabrilla* (Linnaeus, 1758) is a perciform species with minor commercial value, occurring in the Eastern Atlantic and the Mediterranean Sea (Ilhan et al., 2010). Along the Turkish coast of Aegean Sea, an important region for fishing industry, *S. cabrilla* is one of the main species captured by fishing trawlers and the most abundant comber encountered in the Turkish Sea, unlike its congeners (Brown comber *S. hepatus* Linnaeus, 1758; painted comber *S. scriba* Linnaeus, 1758) (Torcu-Koc et al., 2004). This member of Serranidae family was previously registered in Izmir Bay, Central Aegean Sea (Özaydın et al., 2007; Ilhan et al., 2010).

The comber was analysed before in many aspects, concerning: growth and reproduction (Ilhan et al., 2010); feeding habits (Çakir and Koç, 2002); or spatio-temporal patterns of abundance and biomass (Özbek et al., 2016). However, there is still a lack of knowledge regarding the parasites of *S. cabrilla*. As Genc et al. (2005) postulated, since Serranids are predators, the prevalence of infestation in those fish deserves a great deal of attention.

To date, only few parasite species have been reported worldwide on the comber host: the trematode *Lecithochirium musculus* (Paradižnik and Radujković, 2007); the isopods *Ceratothoa steindachneri* (Öktener et al., 2007), *Gnathia* sp. (Alaş et al., 2009) and *Nerocila orbigyni* (Özcan et al., 2015); the anisakid nematodes such as *Anisakis* and *Hysterothylacium* (Kassem and Bowashi, 2015); the copepod *Anchistrotos laqueus* n. sp. (Leigh-Sharpe, 1935) and monogenoidean species *Megalocotyle hexacantha* and *Protolamellodiscus serranelli* (Strona et al., 2010). Among the 14 nominal recognized species of nematodes *Philometra* parasiting the gonads of marine fishes, only *P. serranellicabrillae* Janiszewska, 1949 was described in comber, from the Adriatic Sea and from Mediterranean Sea, in southern Corsica (Moravec et al., 2006).

Until now, in Aegean coast of Turkey no *Philometra* species was founded. Also, there is not yet any evidence of philometrid nematode parasitizing *S. cabrilla* in Turkey. Therefore, this study aimed to investigate for the first time the occurrence of nematode *Philometra* sp. in comber from Aegean coast of Turkey and the aspects of pathogenity within the host.

## MATERIALS AND METHODS

Overall, 83 comber fishes were collected on seasonally basis, during October 2016 and July 2017 along the coasts of Izmir City. Two stations (1) Foça (38°36’58.N; 26°42’59.E) and (2) Sığacık (38°09’19.N; 26°42’29.E) were selected and samplings were carried out by using fishnets of mesh size of 20-24 mm. Each fish individual was examined to evaluate the parasite community.

The total body length of *Serranus cabrilla* was measured and three length-groups were established: 10-14.9, 15-19.9 and 20-25 (cm). Sex of each host was determined at necropsy by macroscopic investigation. During the dissection, internal organs (gastrointestinal tract, liver, kidney, heart, swim bladder, gallbladder and gonads), and body surfaces were examined separately under a dissecting microscope. Fixation, staining and preparation process of the determined parasites was done according to Pritchard and Kruse (1982). The parasites were identified up to

genus level using selected identification keys (Chabaud, 1975; Moravec, 2004, 2006).

Prevalence (Pr %), expressed as the percentage of hosts infected with a particular parasite species or taxonomic group and mean intensity (Int), defined as the total number of individuals of a parasite species per individual infected host were calculated following Bush et al. (1997). Infection rates and statistical analyses were conducted by using Quantitative Parasitology 3.0 web application (Rozsa et al., 2000), Excel programme and SPSS 15. The $\chi 2$-test was performed to test for significant differences between the infection rates over the three length classes. A Kruskal–Wallis test was applied to find significant differences in the mean intensity of the parasite species for host fish size, sex and seasons. Differences were considered to be significant when $p \leq 0.05$.

During the necropsy of comber, gonad samples were collected and fixed in 10% neutral formalin solution for histopathological assessment. After routinely preparation by an automatic tissue processing equipment (Leica ASP300S, Leica Microsystem, Nussloch, Germany), samples were embedded in paraffin and 5μm sections were taken by a Leica RM 2155 rotary microtome (Leica Microsystem, Nussloch, Germany). Then sections were stained with hematoxylin and eosin (HE) and examined under the 40X objective of an Olympus CX41 light microscope. Morphometric evaluation and microphotography was performed using the Database Manual cellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

## RESULTS

Among the total number of 83 specimens of *Serranus cabrilla* examined for parasites, only 12 fish were infected with nematode *Philometra* sp. (Table 1). The prevalence of infection was 14.46% (sexes combined), while the mean intensity of infection during the study was 1.2 parasites per fish. The highest mean intensity (1.3 parasites/fish) was recorded in hermaphrodite host individuals. Overall, the nematode infection was higher in female fishes whilst no worm was found in comber male individuals.

There were significant differences ($P \leq 0.05$) in the infection rates of *S. cabrilla* between length groups, sexes and seasons. The risk factors associated with the highest prevalence of philometrid infection in comber hosts were assessed as follows: hermaphrodite sex of examined individuals, length-group 20-25 cm and spring season (April 2017), respectively. The lowest infection rate was found in autumn (October 2016), and in small comber hosts (15-19.9 cm length-group), respectively. No infection was registered in the warmest month (July 2017). Also, no *Philometra* nematode was detected in the smallest length-group of hosts (10-14.9 cm).

In addition to *Philometra* parasites, in the gonads of some fish individuals were found larvae of anisakid nematodes.

**Table 1.** Seasonal prevalence and intensity of *Philometra* sp. infestation in *Serranus cabrilla* from Izmir Gulf (2016-2017) (N=total number of hosts examined; N'= number of infected fishes; NP=number of collected parasites; Pr=prevalence; Int=mean intensity of infection)

| Parameters | N | N' | Pr (%) | NP | Int |
|---|---|---|---|---|---|
| **Length class (cm)** | | | | | |
| 10.0-14.9 | 16 | 0 | 0 | 0 | 0 |
| 15.0-19.9 | 44 | 3 | 6.82 | 3 | 1 |
| 20.0-25 | 23 | 9 | 39.13 | 11 | 1.2 |
| **Sex** | | | | | |
| Female | 52 | 8 | 15.38 | 9 | 1.1 |
| Male | 6 | 0 | 0 | 0 | 0 |
| Hermaphrodite | 25 | 4 | 16 | 5 | 1.3 |
| **Season** | | | | | |
| October | 27 | 2 | 7.41 | 2 | 1 |
| February | 12 | 1 | 8.33 | 1 | 1 |
| April | 36 | 9 | 25 | 11 | 1.2 |
| July | 8 | 0 | 0 | 0 | 0 |
| Total | 83 | 12 | 14.46 | 14 | 1.2 |

With respect to histopathological findings, it was generally noticed that parasites were attached to the fibrous capsule around the gonads of *S. cabrilla*. Some nematodes were localized inside of the gonads. Hyperemia was the prominent finding. When *Philometra* sp. parasites were localized in the gonadal tissue, they caused a marked inflammatory reaction while slight inflammatory reaction was observed around the parasites disposed the gonads (Fig. 1-3).

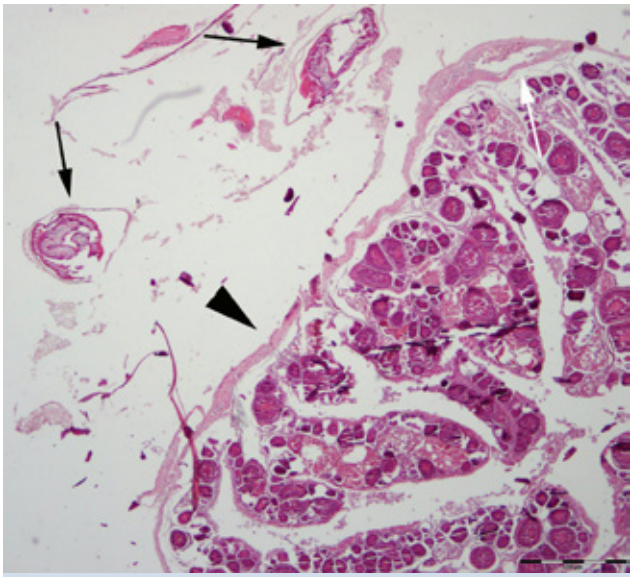

**Figure 1.** Histopathological appearance of the *Philometra* sp. parasites (black arrows) located gonad of *S. cabrilla* (arrow head). Marked hyperemia (white arrow) at the capsular vessels, HE, Bar=200μm.

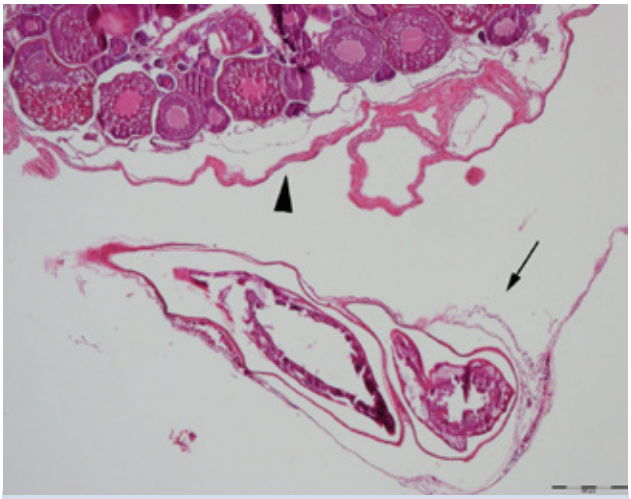

**Figure 2.** A parasite section (arrow) localized outside of the gonad capsule (arrow head), HE, Bar=200μm.

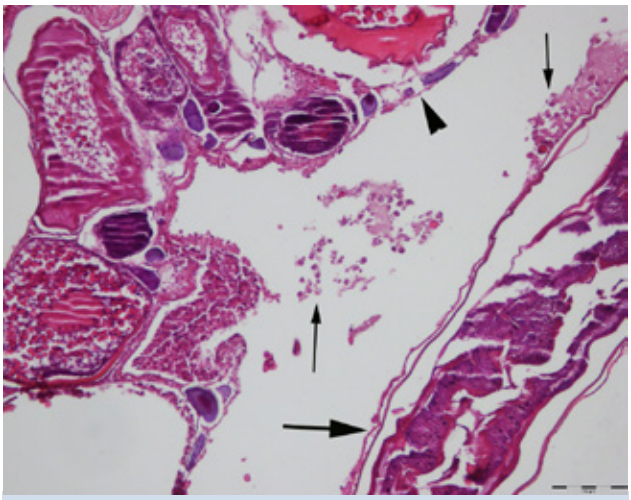

**Figure 3.** Inflammatory cell infiltrations (thin arrows), between *Philometra* sp. parasite (thick arrow) and gonad of *S. cabrilla* (arrow head), HE, Bar=200μm.

## DISCUSSION

The fact that both values of prevalence and mean intensity in nematode infections increase with the body length of the host has been noticed before by various authors who suggested that as fish growth, chances of infection are higher either due to the exposure time or the increase of the internal organs of the fish that a parasite can use to attach itself (Moravec and Scholz, 1995; Al-Zubaidy, 2009; Ahmad et al., 2018). Our data are also consistent with some studies that revealed the relation between the sexually mature fish and occurrence of nematodes, younger fish being less infected or even not infected at all than older fish (Bergmann and Motta, 2004; Szostakowska et al., 2005; Perez et al., 2009). It has been postulated that *S. cabrilla* individuals reach sexual maturity at 3 years old, having an estimated standard length of 15.2 cm at first maturity, and a maximum life span ranged between 4 and 9 years (Torcu-Koc et al., 2004). Taking into account these growth features, we could conclude that the highest prevalence (39.13%) of philometrid infection was reached in sexually mature comber specimens from Izmir Gulf. The reduced percentages of prevalence of *Philometra* infection registered in October and February could be correlated with the fact that these months are out of the reproductive season for the host, since it was assumed that the spawning season on *S. cabrilla* from Mediterranean lasts from April to August, with a mass period in April (García-Días et al., 1997). In agreement with this statement, in the present study, the highest prevalence of parasitic nematodes (25%) was recorded in April. On the one hand, the absence of nematodes in

fish collected during July, in full spawning season can be explained by the type of fish reproduction in the spawning season, when host repetitively expels in the water column its own eggs, along with those belonging to the parasite (Cháves and Oliva, 2011). On the other hand, prevalence of infection categorized as none in July 2018 may be underestimated, as a result of a reduced number of fish collected in this month from the study area and further studies based on larger samples size are needed to clarify this aspect. A possible explanation for the limitations of using a small sample size in the parasitological analyses is given by Marques and Cabral (2007). It was already shown that prevalence and intensity of philometrid infections varies with many factors, including fish species, fishing area, food intake or season (Ali and Afsar, 2018). A relation between the prevalence of infection with *Philometra* adult female worms and the spawning season of its fish host has been suggested by various parasitologists (Genc et al., 2005; Perez et al., 2009; Selvakumar et al., 2015).

Moreover, the lack of parasitic infection in males of *S. cabrilla* could be attributed to the absence of a female-specific physiological trigger responsible for molting larval *Philometra* into adults, as Perez et al. (2009) suggested. The highest prevalence (16%) attributed to hermaphrodite comber individuals may be explained by the reproductive nature of *S. cabrilla*. As García-Días et al. (1997) pointed out, this fish is a synchronous hermaphrodite species, meaning that the ovarian and testicular tissue mature simultaneously.

Factors that influence the prevalence of *Philometra* sp. nematodes have not been documented before in comber host from Aegean Sea. Our data suggest that host body size, sex and season are important risk factors for parasite infection on *S. cabrilla* from Izmir Gulf.

Similar host response was registered by Ali and Afsar (2018), who found that a range of signs and symptoms as inflammation, necrosis and destruction of gonadal tissues are caused by the penetration of nematodes into gonadal wall. Genc et al. (2005) also noticed hyperemia and edema in the ovary of groupers serranid fish from Iskenderun Bay, Northeast Mediterranean Sea, infected with *Philometra lateolabracis.* Study of parasitic nematodes infecting the fish gonad is important, due to the fact that, sometimes, the host is an economically important species. Potential pathogenic effects of parasites belonging to the family Philometridae could result in significant damage to the gonads, including severe changes of reproductive fitness of the host (Cháves and Oliva, 2011; Bakenhaster et al., 2014; Selvakumar et al., 2015). In massive infections, when *Philometra* nematodes feed on ovarian fluid, the parasitic castration and loss of reproductive function in female hosts may occur (Ali and Afsar, 2018).

However, Bakenhaster et al. (2014) have not detected negative effects of *P. floridensis* on the overall health of the red drum *Sciaenops ocellatus*, mentioning that the question regarding how philometrid nematodes affects reproduction in their fish hosts is still a subject to be elucidated.

Concerning the presence of anisakid parasites in the gonads of *Serranus cabrilla*, it must be mentioned that this fish species has been recognize as host for these nematodes by various authors. Thus, Figus et al. (2005) registered adult forms of anisakid genus *Hysterothylacium* from comber fish from southwestern Mediterranean Sea. Kasem and Bowashi (2015) recorded larvae of anisakid parasites belonging to *Anisakis*, *Contracaecum*, *Hysterothylacium* and *Pseudoterranova* taxa in *S. cabrilla* and other marine fishes sampled on Lybian coast.

## CONCLUSIONS

The present paper provides first evidence for the nematode parasites of genus *Philometra* in comber fish from Turkey. Overall, the occurrence of parasite infection was prevalent in the gonads of hermaphrodite and female sexually mature individuals of *Serranus cabrilla*. Our histopathological results show that marked hyperemia occurs when infection caused a marked inflammatory reaction. Since pathological changes in gonadal tissues induced by parasites, both philometrids and anisakids could lead to severe consequences of reproduction of their host, representing also a major concern for fisheries and home cooking, further studies are needed to assess the presence of nematodes and associated zoonoses in fish populations of Izmir Gulf and Aegean coast of Turkey.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Dietary medicinal plants enhance the chemical composition and quality of broiler chicken meat

**T. Tashla[1], N. Puvača[1,*], D. Ljubojević Pelić[2], R. Prodanović[1], S. Ignjatijević[1], J. Bošković[1], D. Ivanišević[1], M. Jahić[1], O. Mahmoud[1], I. Giannenas[3], J. Lević[4]**

[1]*University Business Academy, Faculty of Economics and Engineering Management, Department of Engineering Management in Biotechnology, Novi Sad, Serbia*

[2]*Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia*

[3]*Aristotle University of Thessaloniki, Faculty of Veterinary Medicine, Thessaloniki, Greece*

[4]*University of Novi Sad, Scientific Institute of Food Technology, Novi Sad, Serbia*

**ABSTRACT.** The use of nutritional strategies to improve the quality of meat is a relatively new approach that has emerged at the interface of animal science and food science. The effects of dietary medicinal plants (*Allium sativum* L., *Piper nigrum* L., and *Capsicum annuum* L.) addition to chicken nutrition on quality characteristics of breast and thigh with drumstick meat, as well as caloric value of chicken meat were investigated. Quality measurements included meat sensory (colour, smell, taste, softness, chewiness, juiciness and overall impression), physical (pH, colour – CIE *L*a*b** and drip-loss) and chemical (moisture, protein, fat and ash content) characteristics. Herbs showed significant ($P < 0.05$) influence in altering most examined quality parameters of chicken meat, especially when adding red hot pepper. Caloric value of chicken meat was improved which makes garlic, black pepper and hot red pepper valuable natural feed additives in improvement of meat quality as well as a natural growth promoter. In conclusion, herbs had positive influence on chicken meat quality, however the knowledge of their mode of action is still limited and thus requires further investigation.

***Keywords:*** Medicinal plants, nutrition, meat quality, sensory, physical characteristics

*Corresponding Author:*
Nikola Puvača, University Business Academy, Faculty of Economics and Engineering Management, Department of Engineering Management in Biotechnology, Cvećarska 2, 21000 Novi Sad, Serbia
E-mail address: nikola.puvaca@gmail.com

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## INTRODUCTION

In last decade, consumers have become more concerned about the processed food they consume. Synthetic preservatives, which have been used in food industry, may lead to negative health consequences. The use of synthetic compounds have significant drawbacks, such as increasing cost, handling hazards, concerns about residues on food and threat to human environment. Therefore, there has been increasing interest to replace synthetic with natural, effective and nontoxic compounds (Puvača, 2018). Those, in the first place are extracts and essential oils (Giannenas et al., 2013; Puvača et al., 2019), spices (Puvača et al., 2015; Popović et al., 2018) and medicinal plants (Kostadinović et al., 2015; Puvača et al., 2018). As natural foodstuffs, spices and medicinal plants appeal to all who question safety of synthetic food additives and demand high quality products that at the same time are safe and stable. Medicinal plants have been extensively used in the therapy of some diseases for a long time. Interest in plants, plant extracts and derived phytochemicals as components of livestock feedstuffs has increased during the last years (Puvača et al., 2013). Moreover, medicinal plants and aromatic plants possess many antioxidants which are effective in preventing oxidative changes and, thus, can minimize off-odour production in meat (Najafi and Torki, 2010). The use of nutritional strategies to improve the quality of meat is a relatively new approach that has emerged at the interface of animal science and food science (Džinić et al., 2015; Puvača et al., 2016; Spasevski et al., 2018). Nutritional approaches are often more effective than direct addition of the additive to meat since the compound is preferably deposited where it is most needed (Govaris et al., 2004). As in other animal species, the physical quality of broiler meat is of major importance, since broiler chicken meat is nowadays usually consumed as cuts or as processed products rather than as whole carcasses. Physical quality refers to several meat properties, including pH and colour values. Meat quality is closely related to the decrease in muscle pH *postmortem*. Rapid *postmortem* decline in pH results in PSE (pale, soft and exudative) meat with a pale aspect and reduced water-holding capacity (Owens et al., 2000; Puvača and Stanaćev, 2011). The variations in colour of broiler breast meat fillets are significant correlated with muscle pH and extremes in colour variations (Khalafalla et al., 2011). Low ultimate pH results in "acid meat", with similar defects to those of PSE meat (Barbut, 1997), while high ultimate pH leads to DFD (dark, firm and dry) meat with dark colour and poor storage quality (Allen et al., 1997; Laudadio and Tufarelli, 2011). Beside physical meat quality, the economic importance is also related to sensory and chemical characteristics of meat which should not be forgotten. To ensure optimum quality, it is necessary to consider the entire production chain from farm to fork (Khalafalla et al., 2011). Many studies focused on the impact of many dietary supplemental components with the aim to find more efficient alternatives or combinations of different alternatives for maintaining health and improving performance of poultry and meat quality and safety, without antibiotics residues.

Therefore, the aim of this study was to investigate the influence of three different medicinal plants (garlic, black pepper and hot red pepper) in broiler diet on meat sensory, physical and chemical characteristics.

## MATERIALS AND METHODS

### Animal housing and nutrition

Biological tests were carried out under production conditions at the experimental farm "Pustara" of the Faculty of Agriculture in Novi Sad, Serbia. All experimental procedures have been approved by the competent Veterinary Authority according to the National legislation (Presidential Degree 56/2013 on harmonization of the Directive 2010/63/EU on the protection of animals used for scientific purposes) under registered number I-2015-02. At the beginning of the experiment, a total of 1200 one-day old Hubbard broilers strain, of mixed sex, were distributed into eight dietary treatments with four replicates each (Table 1).

Every dietary treatment included 150 chickens, which were divided in four pens (37-38 chicken per pen). Feeding program included three-phase diet as starter, grower and finisher, respectively. For the first 14 days, during the acclimation period, chicks were fed with starter diet, then birds were fed with grower diet for the next 21 days and then for the last 7 days of fattening period with finisher diet. During the whole trial feed and water were provided *ad libitum.* Microclimate conditions were regularly monitored. Rearing and housing condition were previously described in detail by Puvača et al. (2015), while the nutrition of the chickens were previously described in detail by Puvača et al. (2016).

**Table 1.** Experimental design with chickens and dietary treatments, g/100g

| Experimental treatments | Concentration of additives in chicken diets | | | |
|---|---|---|---|---|
| | Additive | Starter | Grower | Finisher |
| | | 1 – 14 days | 15 – 35 days | 36 – 42 days |
| T1 | Control treatment | 0.0 | 0.0 | 0.0 |
| T2 | Garlic powder | 0.0 | 0.5 | 0.5 |
| T3 | Garlic powder | 0.0 | 1.0 | 1.0 |
| T4 | Black pepper powder | 0.0 | 0.5 | 0.5 |
| T5 | Black pepper powder | 0.0 | 1.0 | 1.0 |
| T6 | Hot red pepper powder | 0.0 | 0.5 | 0.5 |
| T7 | Hot red pepper powder | 0.0 | 1.0 | 1.0 |
| T8 | Mixture of garlic, black pepper and hot red pepper (1:1:1) | 0.0 | 0.5 | 0.5 |

**Sample collections**

At the end of 42nd day of the experiment, 12 broiler chicks, six male and six female of an average body weight of each treatment group were selected for meat quality evaluations. Before slaughtering broiler chickens were starved for 12 h, and afterword slaughtered were processed by bloodletting, scalding, plucking and evisceration and chilled. Upon slaughter, dressed cold carcasses were dissected into primal cuts such as breast, thighs with drumsticks, wings, back, head, neck and legs following the method prescribed by the Regulation on Poultry Meat Quality (Official Gazette of the SFRY No. 1/81 and 51/88). After 24 hours post-mortem breast (*Musculus pectoralis*) and thigh with drumstick (*Tibialis anterior* and *Biceps femoris*) were further analysed for their sensory, physical and chemical characteristics.

**Sensory analyses**

Sensory assessment was conducted at the laboratory of Scientific Institute of Food Technology in Novi Sad, Serbia, equipped in accordance with the ISO standard (8589:2012) of Sensory analyse - General guidance for the design of test rooms. Five trained panellists with expertise in sensory evaluation of meat and meat products evaluated selected properties for breast (colour, smell, taste, softness, chewiness and juiciness) and thigh with drumstick (colour and smell and taste) meat. Sensory analyses were evaluated using a point system of analytical descriptive tests with a scale from 1 to 5, for the colour (1=very bad; 2=bad, 3=good; 4=very good; 5= extremely good), for the smell and taste (1=extremely unpleasant; 2=unpleasant; 3=insufficiently pleasant; 4=pleasant, good; 5=extremely pleasant, excellent), for softness (1=moderately tender; 2=tightly; 3=moderately tightly; 4=moderately soft; 5=soft), for chewiness (1=moderately toughened; 2=poor toughened; 3=chewable; 4=moderately gentle; 5=gentle), for juiciness (1=dry; 2=moderately dry; 3=moderately juicy; 4=juicy; 5=extremely juicy) and for overall impression (1=sufficient; 2=suit; 3=good; 4=very good; 5=excellent). Panellists were randomly supplied with thermally processed meat samples. Between each sample evaluation, panellists were cleaning their palate with distilled water, bread or apple. Between each repetition period of samples evaluation, 1 h was awarded to panellists for senses resting.

**Physical analyses**

The pH values of breast meat and thigh with drumstick were measured using the portable pH meter (Consort C931, Turnhout, Belgium) equipped with an insertion glass combination electrode (Mettler Toledo Greifensee, Switzerland). Means of twelve measurements were presented. Colour measurements of breast and thigh with drumstick meat were carried out using photo-colorimeter Minolta Chroma Meter CR-400 (Minolta Co., Ltd., Osaka, Japan). Colour characteristics are given in the CIE $L^*a^*b^*$ system ($L^*$ = lightness; $a^*$ = redness and $b^*$ = yellowness). Two measurements were taken on surfaces and cut places of meat samples for each treatment. Data presented are means of 24 measurements. For drip-loss of heat processing determination, samples of meat were measured before heat processing and after the treatment samples were cooled at room temperature for 1 h, and measured in order to determine the loss.

**Chemical analyses and meat caloric value**

Meat chemical characteristics were determined according to the ISO recommended standards for moisture, protein, fat and ash contents. Data presented are means of twelve measurements.

The caloric value of 100 g of white and dark meat by influence of dietary spice plants addition to chicken daily nutrition was calculated according to the following equation:

Caloric value (kcal) = (protein (g/100g) × 4 kcal) + (fat (g/100g) × 9 kcal)

**Statistical analyses**

Statistical analyses were conducted using statistical software program Statistica 13 for Windows, to determine if variables differed between treatments. Significant effects were further evaluated using analysis of variance (ANOVA), least square means (LSM) and standard errors of least square means ($SE_{LSM}$). Fisher's LSD *post-hoc* multiple range test with Bonferroni corrections were used to ascertain differences among treatments. A significance level of $P<0.05$ was used.

## RESULTS AND DISCUSSION

Results in this paper are presented in a form of tables with the least square means and standard errors of least square means, while in available literature there is no sufficient data on meat quality regarding the medicinal plants we used, which makes this investigation a novelty in field of chicken meat quality. Therefore, discussion will go in way of presenting of our findings compared with published works regarding the sensory, physical and chemical quality of broiler meat.

**Sensory characteristics**

Results for sensory characteristics (colour, smell, taste, softness, chewiness, juiciness and overall impression) of breast and thigh with drumstick meat of broiler chickens fed with different dietary spice addition are presented in Table 2. Supplementing medicinal plants in broiler ration such as garlic, black pepper and hot red pepper and their combined mixture led to significant differences ($P < 0.05$) in meat quality characteristics. The T1 muscles of breast meat had the significantly ($P < 0.05$) lowest visual colour score compared to the other experimental treatments. The highest visual colour score of thigh with drumstick meat was recorded in treatment with 1.0 g/100g (T7, 4.9) of hot red pepper with significant ($P < 0.05$) differences compared to treatments T2, T4 (4.5) and T5, T6 (4.6), respectively. When it comes to the scores of breast meat smell, significant differences ($P > 0.05$) were absent, but the highest score of 4.9 was recorded in thigh with drumstick meat in treatment T5 followed by 4.8 (T3, T7, T8), 4.7 (T1), 4.6 (T4), 4.4 (T2) and 4.3 (T6), respectively. Similar tendency was observed in breast meat taste. As it was the case with the breast meat smell, the same highest score of meat softness was observed with the addition of 1.0 g/100g of hot red pepper in treatment T7, which get the score of soft meat. The lowest score of softness was recorded in breast meat of chickens fed 1.0 g/100g of garlic powder, but again higher then score point of 3, resulting as moderately soft which is very acceptable by consumers. Gentle and moderately gentle scores of breast meat was recorded in control treatment (T1), 1.0 g/100g hot red pepper (T7), 0.5 g/100g black pepper and hot red pepper (T4, T6) with significant ($P < 0.05$) differences compared to treatments T2 and T8, respectively. Hot red pepper at both concentrations have positively influenced on the breast meat juiciness with the highest scores of 4.5 and 4.4, respectively. When it comes to overall impression, dietary of hot red pepper showed the highest scores of sensory meat quality, while the lowest was observed in garlic treatment groups with an average score of 4.3.

**Table 2.** Effect of garlic, black pepper and hot red pepper dietary supplementation on sensory quality of broiler chicken breast and thigh with drumstick (Least squares means)

| Experimental treatments | | Quality parameters of breast meat | | | | | | | Quality parameters of thigh with drumstick | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Colour | Smell | Taste | Softness | Chewiness | Juiciness | Overall impression | Colour | Smell and taste |
| T1 | LSM | $4.9^{b}$ | $4.8^{a}$ | $4.7^{a}$ | $4.5^{abc}$ | $4.5^{a}$ | $4.3^{ab}$ | $4.5^{ab}$ | $4.7^{abc}$ | $4.7^{abc}$ |
| T2 | LSM | $5.0^{a}$ | $4.7^{a}$ | $4.5^{ab}$ | $4.4^{abcd}$ | $4.2^{ab}$ | $4.0^{bd}$ | $4.3^{b}$ | $4.5^{bc}$ | $4.4^{cd}$ |
| T3 | LSM | $5.0^{a}$ | $4.8^{a}$ | $4.6^{ab}$ | $4.1^{d}$ | $4.2^{ab}$ | $3.8^{d}$ | $4.3^{ab}$ | $4.7^{ac}$ | $4.8^{ab}$ |
| T4 | LSM | $5.0^{a}$ | $4.9^{a}$ | $4.8^{a}$ | $4.5^{abc}$ | $4.4^{ab}$ | $4.2^{abc}$ | $4.5^{ab}$ | $4.5^{c}$ | $4.6^{bcd}$ |
| T5 | LSM | $5.0^{a}$ | $4.7^{a}$ | $4.5^{ab}$ | $4.3^{bcd}$ | $4.3^{ab}$ | $4.3^{ab}$ | $4.4^{ab}$ | $4.6^{bc}$ | $4.9^{a}$ |
| T6 | LSM | $5.0^{a}$ | $4.7^{a}$ | $4.5^{ab}$ | $4.6^{ab}$ | $4.4^{ab}$ | $4.5^{a}$ | $4.6^{a}$ | $4.6^{bc}$ | $4.3^{d}$ |
| T7 | LSM | $5.0^{a}$ | $4.8^{a}$ | $4.7^{ab}$ | $4.7^{a}$ | $4.5^{a}$ | $4.4^{a}$ | $4.6^{ab}$ | $4.9^{a}$ | $4.8^{ab}$ |
| T8 | LSM | $5.0^{a}$ | $4.7^{a}$ | $4.4^{b}$ | $4.2^{cd}$ | $4.0^{b}$ | $3.9^{cd}$ | $4.3^{ab}$ | $4.8^{ab}$ | $4.8^{ab}$ |
| Pooled $SE_{LSM}$ | | 0.01 | 0.11 | 0.11 | 0.12 | 0.14 | 0.14 | 0.10 | 0.1 | 0.08 |

Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)

Sensory analysis is one of the oldest means of meat quality control which also allowing manufacturers to identify, understand and respond to consumer preferences more effectively (Saha et al., 2009), helping manufacturers to increase competition on the market (Ponte et al., 2004). In our experiment, the sensory quality of meat shows very high scores for smell, juiciness and overall impression, which leads to conclusion that the addition of medicinal plants didn't have adverse effect on meat quality and that the amount of added plants in chicken diet was adequate. Contrary to our results, investigation of Džinić et al. (2015) evaluated the sensory quality of breast chicken meat with the addition of 2.0% of garlic powder showing characteristic taste and pungent smell of garlic from broiler feed which was transferred to meat. This characteristic flavour can be both non-beneficial and beneficial depending of consumers affinities and demands. In the same investigation, the addition of garlic led to excellent meat quality regarding the juiciness and tenderness of breast meat, in accordance with our study. Similar results were obtain by Popović et al. (2016) using selected essential oils in broiler diet on sensory meat quality, which reviled significant influence of this phytobiotic in altering of taste, juiciness and tenderness of chicken meat. Addition of 30 mg/kg of *Macleaya cordata* (Zdunczyk et al., 2010) which is from same alkaloid family as black pepper shows overall intensive smell for breast meat without any atypical smell. As for the breast meat, similar results were obtained for the thigh meat sensory evaluation. According to our findings, also the study of Waskar et al. (2011) reported that supplementation of polyherbal feed supplement in basal diet was effective in improving overall meat quality attributes such as fillet and tender yield, sensory meat characteristics, organoleptic cooked meat parameters, overall palatability and acceptability of meat. The natural products such as garlic, black pepper and hot red pepper did not have any residual or adverse effect on eating and cooking quality of meat, and hence resulted safe for usage. This study have confirmed that the direct influence on meat quality have nutrition of chickens what is in accordance with previous mentioned findings.

**Physical characteristic**

Results for meat physical characteristics (pH value, colour – CIE *L*a*b** values and drip-loss of heat treatment) of all eight dietary treatments are presented in Tables 3 and 4, respectively.

Slightly small numerical average values of breast meat pH with significant ($P < 0.05$) differences (ranged from 5.5 to 5.7) which are smaller than 0.3 can be explained as a possible statistical error (Table 3). One of the basic tenets of meat science is that accelerated or extended *postmortem* glycolysis may cause the development of PSE. There are implicit interrelationships between temperature and pH because glycolysis is exothermic, and the effects of pH are severe when a carcass is still near body temperature. Meat with a low pH appears pale because it scatters more light back to the observer than meat with a high pH which appears dark because it transmits more light into its depth than meat with a low pH. In this experiment the lowest pH of 5.5 was recorded in breast meat of chicken in dietary treatment T4 fed 0.5 g/100g of black pepper powder, while the highest pH (5.7) was observed in treatments T2 and T6 with dietary addition of 0.5 g/100g of garlic and hot red pepper powder.

**Table 3.** Effect of garlic, black pepper and hot red pepper dietary supplementation on physical parameters of breast meat (Least squares means)

| Experimental treatments | | Physical parameters of breast meat | | | | |
|---|---|---|---|---|---|---|
| | | pH | Colour values | | | Drip-loss |
| | | | L* | a* | b* | |
| T1 | LSM | $5.6^{ab}$ | $57.4^{a}$ | $2.5^{a}$ | $5.8^{a}$ | $22.9^{cd}$ |
| T2 | LSM | $5.7^{ab}$ | $57.0^{ab}$ | $2.5^{a}$ | $5.4^{a}$ | $31.4^{a}$ |
| T3 | LSM | $5.6^{ab}$ | $54.3^{ab}$ | $2.9^{a}$ | $6.3^{a}$ | $29.9^{a}$ |
| T4 | LSM | $5.5^{b}$ | $56.5^{ab}$ | $2.0^{a}$ | $4.2^{a}$ | $26.1^{b}$ |
| T5 | LSM | $5.6^{ab}$ | $53.4^{ab}$ | $2.3^{a}$ | $4.3^{a}$ | $23.1^{cd}$ |
| T6 | LSM | $5.7^{a}$ | $53.1^{b}$ | $2.3^{a}$ | $3.9^{a}$ | $20.5^{d}$ |
| T7 | LSM | $5.6^{ab}$ | $57.1^{ab}$ | $2.4^{a}$ | $6.3^{a}$ | $24.3^{bc}$ |
| T8 | LSM | $5.6^{ab}$ | $55.6^{ab}$ | $2.4^{a}$ | $4.4^{a}$ | $31.1^{a}$ |
| Pooled $SE_{LSM}$ | | 0.04 | 1.38 | 0.49 | 0.87 | 0.93 |

Means in the same column with a common superscript letter are not significantly different ($P < 0.05$)

When it comes to a CIE *L*a*b** colour values, from the results given in Table 3, it can be observed that the differences in *a** (redness) and *b** (yellowness) colour system is present between dietary treatments (*a**: 2.0-2.9; *b**: 3.9-6.3) but without significant differences (P > 0.05). Colour values for *L** (lightness) show significant differences (P < 0.05) among dietary treatments. The lowest value for lightness was observed in breast meat of chickens on dietary treatment including 0.5 g/100g of hot red pepper, while the highest value for the lightness of breast meat was observed in control treatment. Based on the results of pH and colour values, breast meat could be classified as PSE, but if we take in account the scores of sensory analyses of meat, and that the white meat is in question it could be classified as meat of normal quality, with the significant influence of dietary spice plants addition.

Heat processing of meat had a significant influence on meat drip-loss (P < 0.05). Hot red pepper in treatment T6 showed the highest positive influence, where the lowest drip-loss of meat after heat treatment was observed (20.5%). In the other experimental dietary treatments meat drip-loss ranged from 23.1 to 31.1%, with the highest value observed in treatment with the addition of 0.5 g/100g of garlic powder.

Data on physical quality of chicken red meat (thigh with drumstick) are shown in Table 4. From our findings it can be noticed a very small average differences in pH values, but with significant differences (P < 0.05), as it was case with breast meat. Values of pH ranged from 5.9 to 6.0, so the thigh with drumstick can be classified as a normal quality meat. The highest value of redness (*a**) was observed in treatment with the addition of 1.0 g/100g of hot red pepper with significant (P < 0.05) differences compared to other experimental dietary treatments. The lowest *a** value was recorded in treatment T4 with the addition of black pepper powder. Contrary to white meat, significant differences (P < 0.05) were observed and in *b** colour values of thigh and drumstick meat, which ranged from 4.2 (T3) to 6.2 (T2), respectively. The highest lightness (61.0) combined with high redness (3.6) was observed in meat of control treatment (P < 0.05) and it could be classified as reddish, soft and exudative (RSE) meat, while the addition of spices especially hot red pepper had positive influence of red meat, which could be classified as normal.

Dietary addition of hot red pepper also shows its positive influence on meat drip-loss after heat treatment. Experimental treatment T7 recorded lowest drip-loss value (27.3%).

**Table 4.** Effect of garlic, black pepper and hot red pepper dietary supplementation on physical parameters of thigh with drumstick (Least squares means)

| Experimental treatments | | Physical parameters of thigh with drumstick | | | | |
|---|---|---|---|---|---|---|
| | | pH | Colour values | | | Drip-loss |
| | | | L* | a* | b* | |
| T1 | LSM | $6.0^{ab}$ | $61.0^{a}$ | $3.6^{ab}$ | $5.4^{ab}$ | $30.2^{ab}$ |
| T2 | LSM | $6.0^{ab}$ | $60.7^{ab}$ | $2.9^{bc}$ | $6.2^{a}$ | $31.2^{a}$ |
| T3 | LSM | $6.0^{ab}$ | $57.2^{bc}$ | $2.4^{bc}$ | $4.2^{ab}$ | $31.6^{a}$ |
| T4 | LSM | $5.9^{b}$ | $60.3^{abc}$ | $2.3^{c}$ | $4.4^{ab}$ | $32.6^{a}$ |
| T5 | LSM | $6.0^{ab}$ | $57.0^{c}$ | $2.4^{bc}$ | $4.5^{ab}$ | $31.1^{a}$ |
| T6 | LSM | $6.0^{a}$ | $58.9^{abc}$ | $2.9^{bc}$ | $3.6^{b}$ | $33.7^{a}$ |
| T7 | LSM | $5.9^{ab}$ | $58.2^{abc}$ | $4.2^{a}$ | $5.5^{ab}$ | $27.3^{b}$ |
| T8 | LSM | $5.9^{ab}$ | $59.0^{abc}$ | $3.2^{abc}$ | $5.3^{ab}$ | $31.9^{a}$ |
| Pooled $SE_{LSM}$ | | 0.04 | 1.21 | 0.44 | 0.74 | 1.2 |

Means in the same column with a common superscript letter are not significantly different (P < 0.05)

Considering the glycolytic process, initial pH value provides best information for physiological meat quality. Highly accelerated as well as accelerated pH decline are related to a light meat colour and poor juice retention, which in the majority of cases is the same for pH values ranging from 5.8 to 6.0 concerning exudative meat composition. Besides pH value many other sensory, instrumental and biophysical methods are involved in determination of chicken meat quality (Damez and Clerjon, 2008). During recent years, deviations in meat quality which did not show typical characteristics of PSE and DFD were repeatedly reported. There was acceptable colour and increased wateriness (RSE) as well as pale colour and good juice retention (PFN). Of especially interest is RSE meat, as drip-loss means a loss in weight and therefore economic losses. In our experiment, pH value of chickens breast meat ranged from 5.5 to 5.7 and in thigh and drumstick between 5.9 and 6.0 with observed significant (P < 0.05) differences, while the Qiao et al. (2001) in their investigation reported pH values of

the lighter than-normal, normal and darker-than-normal groups with 5.81, 5.96, and 6.23, respectively, which were significantly different from each other. Stanaćev et al. (2011) recorded pH value of 5.6 in meat from chickens fed 2% of garlic powder. According to Karunanayaka et al. (2016), meat with the pH higher than 5.8 is classified as normal meat quality. Several researchers have demonstrated a significant relationship between raw meat colour and raw meat pH (Allen et al., 1997, 1998; Fletcher, 1999). Barbut (1993) reported that lightness (*L**) had the highest correlation of the *L*, a*, b** colour values with PSE conditions. In our experiment, the dietary addition of garlic, black pepper and hot red pepper had influence on broiler chicken meat colour. The relationship between muscle pH, colour, and meat quality in red meat species is well established. As noted earlier, the relationship between poultry meat colour and pH has also been well documented, but the relative influence on poultry meat quality is not as well established as in the extremes of PSE and DFD conditions in pork and beef (Qiao et al., 2001). Positive influence of medicinal plants addition in broiler chicken diet on meat quality was also observed by Savković et al. (2008) using a mixture of different spice and aromatic herbs. Hot red pepper in the current experiment showed the highest positive influence with the lowest drip-loss of breast meat after heat treatment (20.5%), while the higher addition of same spice (T7) in thigh with drumstick also showed positive influence and lowest drip-loss of 27.3%. Allen et al. (1998) reported that initial and tumbled *L** values are correlated positively with drip-loss and cook-loss of chicken meat. Their results were in agreement with the previous findings of Barbut (1993), who observed a high correlation between *L** and cooking loss, whereas the Allen *et al.* (1998) found no correlation between raw meat pH and drip-loss or cook-loss. In our trial, the highest drip-loss of breast meat was observed in treatment T2 with *L** of 57.0 and in treatment T6 with *L** of 58.9 of red meat, which is in agreement with earlier findings.

**Chemical characteristics and caloric characteristic of chicken meat**

From the results given in Table 5, it can be observed no significant (P > 0.05) differences in moisture content in both breast and thigh with drumstick meat. Moisture in white meat ranged from 71.8 to 73.9 g/100g and 72.5 to 74.8 g/100g in red meat, respectively. The highest content of protein (24.0 g/100g) was observed in treatment T5 with significant (P < 0.05) differences compared to treatments T2 (22.3), T1 (22.1) and T8 (21.8 g/100g), respectively. The lowest amount of fat was observed in treatment T5 (0.16 g/100g) what was expected because of the negative correlations between the content of protein and fat. The highest fat content was recorded in control treatment T1 (0.55 g/100g) with significant (P < 0.05) differences compared to the other treatments. Significant differences between the groups (P < 0.05) were found in for the ash content of both breast meat and thigh with drumstick meat. The highest content of protein (20.6 g/100g) in thighs with drumsticks was observed in dietary treatment with addition of hot red pepper (T7). Interesting is that the same treatment also contains highest content of fat (3.86 g/100g). This could be explained by the influence of capsaicin as the active compound in hot red pepper on the metabolism of fat and the highest utilisation from the feed which is incorporated in the body. The lowest content of protein in meat, as in the breast meat was observed in control treatment (18.6 g/100g) with the fat content of 2.59 g/100g, which could be a sign of positive influence of dietary medicinal plants addition to alteration of chicken meat nutritive quality.

**Table 5.** Effect of garlic, black pepper and hot red pepper dietary supplementation on chemical characteristic of breast meat and thigh with drumstick (Least squares means)

| Experimental treatments | | Quality parameters of breast meat | | | | Quality parameters of thigh with drumstick | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Moisture | Protein | Fat | Ash | Moisture | Protein | Fat | Ash |
| T1 | LSM | 73.8$^{a}$ | 22.1$^{b}$ | 0.55$^{a}$ | 1.12$^{bc}$ | 74.7$^{a}$ | 18.6$^{b}$ | 2.59$^{e}$ | 1.00$^{b}$ |
| T2 | LSM | 73.4$^{a}$ | 22.3$^{b}$ | 0.48$^{b}$ | 1.19$^{ab}$ | 74.3$^{a}$ | 18.7$^{b}$ | 3.74$^{a}$ | 1.06$^{a}$ |
| T3 | LSM | 73.9$^{a}$ | 22.6$^{ab}$ | 0.30$^{c}$ | 1.18$^{ab}$ | 72.7$^{a}$ | 19.8$^{ab}$ | 2.79$^{d}$ | 1.09$^{a}$ |
| T4 | LSM | 73.3$^{a}$ | 22.6$^{ab}$ | 0.28$^{c}$ | 1.16$^{abc}$ | 74.2$^{a}$ | 19.4$^{ab}$ | 2.99$^{c}$ | 1.00$^{b}$ |
| T5 | LSM | 72.3$^{a}$ | 24.0$^{a}$ | 0.16$^{d}$ | 1.24$^{a}$ | 72.5$^{a}$ | 20.0$^{a}$ | 2.26$^{f}$ | 1.07$^{a}$ |
| T6 | LSM | 71.8$^{a}$ | 22.7$^{ab}$ | 0.49$^{b}$ | 1.17$^{ab}$ | 74.3$^{a}$ | 19.5$^{ab}$ | 3.24$^{b}$ | 1.08$^{a}$ |
| T7 | LSM | 72.6$^{a}$ | 21.8$^{b}$ | 0.18$^{d}$ | 1.06$^{c}$ | 73.3$^{a}$ | 20.6$^{a}$ | 3.68$^{a}$ | 1.06$^{a}$ |
| T8 | LSM | 72.4$^{a}$ | 22.5$^{ab}$ | 0.27$^{c}$ | 1.17$^{ab}$ | 74.8$^{a}$ | 19.7$^{ab}$ | 2.13$^{f}$ | 1.08$^{a}$ |
| Pooled $SE_{LSM}$ | | 0.81 | 0.53 | 0.02 | 0.02 | 0.81 | 0.44 | 0.04 | 0.02 |

Means in the same column with a common superscript letter are not significantly different (P < 0.05)

Moisture content of white and red meat was almost equal among dietary groups. Similarly, research reported that broiler chickens feed supplementation with Echinacea (*Echinacea purpurea*), garlic (*Allium sativum*) and ginger (*Zingiber officinale*) resulted in no effect on meat moisture content. In our experiment, the highest protein content of breast (24.0 g/100g) and thigh with drumstick (20.6 g/100g) meat was observed in treatments T5 and T6, respectively. From this fact, it can be noticed that the dietary addition of black pepper and hot red pepper to chicken ration led to significant improvement of meat quality. In a previous research of Stanaćev et al. (2011), dietary garlic powder addition to chickens diet at level of 2.0 g/100g, resulted in significant differences in protein content (22.9 g/100g) in breast meat compared to control diet (21.8 g/100g). The lowest protein content of 18.6 g/100g was observed in red meat from control treatment. Souza et al. (2011) reported that protein content of chicken breast meat ranged from 22.48 to 22.61 g/100g without significant differences. Content of protein in chicken breast meat in range from 20.7 to 32.1% was reported by Mohammed (2013) with energy value between 160.0 and 212.0 kcal. In our experiment, the energetic value of breast meat was influenced by dietary medicinal plants addition and it ranged from 92.5 kcal (T8) to 97.5 kcal (T5). Onibi et al. (2009) reported significant influence of spice and herbs in broiler nutrition on fat content, where the thigh muscle had the highest fat content (82.9 g/kg), followed by drumstick muscle (66.9 g/kg) and lowest for breast muscle (49.1 g/kg). Lowest fat content of breast meat in the current experiment was recorded when birds fed 1.0 g/100g of black pepper powder (0.16 g/100g) and 2.13 g/100g in red meat of chicken fed with mixture of garlic, black pepper and hot red pepper powder at 0.5 g/100g. Similarly, fat deposition has been reported to be higher in red meat than in breast meat (Onibi, 2006). Breast meat fat content was affected by the interaction between sex and genetic strain, with males presenting the highest values as found by Souza et al. (2011). On the other hand, Lonergan et al. (2003) reported higher fat content values in females as influenced by the interaction between sex and genetic group fed with the same dietary mixtures. Addition of black pepper powder had high influence on mineral content of breast meat in our experiment. Significantly higher ash content of chicken meat was reported by Mohammed (2013) who is in accordance with our results.

Addition of medicinal plants to broiler chickens diet resulted in significant ($P < 0.05$) differences in meat caloric value (Table 6). Chickens fed with 1.0 g/100g of black pepper had the highest energetic value (97.7 kcal), while the chickens fed with the same amount of hot red pepper showed the lowest energetic value (88.9 kcal) of breast meat ($P < 0.05$). Small differences between the other treatments were observed ($P > 0.05$). When it comes to caloric value of red meat, conversely to white meat, the addition of hot red pepper (T7) influenced in high energetic value (115.5 kcal) of meat with statistically significant ($P < 0.05$) differences compared to all other treatments. The lowest energetic value of red meat was recorded in treatments T5 and T8, while the high energetic value (108.5 kcal) was recorded in red meat of chickens fed with dietary addition of garlic at the level of 0.5 g/100g.

When it's come to energetic value of chicken meat, it can be observed that the addition of spices to chicken nutrition had significant influence on altering caloric value of meat.

**Table 6.** Effect of garlic, black pepper and hot red pepper dietary supplementation on caloric values of chickens breast meat and thigh with drumstick (Least squares means)

| Type of meat | | Dietary treatments | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 |
| White meat | LSM | $93.5^{ab}$ | $93.8^{ab}$ | $93.4^{ab}$ | $93.2^{ab}$ | $97.7^{a}$ | $95.4^{ab}$ | $88.9^{b}$ | $92.5^{ab}$ |
| | $SE_{LSM}$ | 2.26 | 2.26 | 2.26 | 2.26 | 2.26 | 2.26 | 2.26 | 2.26 |
| Red meat | LSM | $98.0^{c}$ | $108.5^{b}$ | $104.5^{bc}$ | $104.5^{bc}$ | $100.6^{cd}$ | $107.1^{bd}$ | $115.5^{a}$ | $98.2^{c}$ |
| | $SE_{LSM}$ | 2.23 | 2.23 | 2.23 | 2.23 | 2.23 | 2.23 | 2.23 | 2.23 |

Means in the same row with a common superscript letter are not significantly different ($P < 0.05$)

## CONCLUSIONS

Based on our findings, it can be concluded that the dietary addition of garlic, black pepper and hot red pepper to broiler diet showed significant influence in improvement of sensory, physical and chemical characteristics of meat. It is important that meat from chicken fed with medicinal plants resulted in higher energetic value which is of practical importance for human nutrition. Moreover, it can be concluded that administrative and technological as well as nutritional and sensory quality of chicken meat can only be reached with proven feed supplements, because of not all of the additives may have beneficial effects on meat quality, but can have the opposite tendency. From this trial, the used medicinal plants showed significant effect on chicken meat quality, but the knowledge of their use is still limited, and thus further investigation is still necessary.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

# Assessment of plasma nitric oxide concentration and erythrocyte arginase activity in dairy cows with traumatic reticuloperitonitis

**A. Kirbas[1*], E. Baydar[2], F.M. Kandemir[3]**

[1]*Department of Internal Medicine, Faculty of Veterinary Medicine, Ataturk University, Erzurum/Turkey*

[2]*Department of Internal Medicine, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir/Turkey*

[3]*Department of Biochemistry, Faculty of Veterinary Medicine, Ataturk University, Erzurum/Turkey*

**ABSTRACT.** The aim of this study was to evaluate plasma nitric oxide (NO) concentrations, erythrocyte arginase (ARG) activity, plasma fibrinogen (Fb) and serum iron (Fe) levels and some biochemical parameters in dairy cows with traumatic reticuloperitonitis (TRP). The animal material of the study consisted of 14 Swiss Brown cows diagnosed with TRP (TRP group) between 4-8 years old brought to Firat University Animal Hospital Clinics and 14 healthy Swiss Brown cows (control group) aged 4-8 years obtained from dairy farms in different regions. Blood samples were taken from the *vena jugularis* of the animals. Concentrations of plasma NO, Fb, erythrocyte ARG activity, and some biochemical markers were determined after the serum and plasma of the receiving blood were separated. While the NO (318.9±5.8 vs. 270.3±9.6 μmol/L) concentrations of the TRP group were found to be significantly higher than the control group (P<0.001), the erythrocyte ARG activity (29.5±0.5 vs. 35.2±1.0 U/hb) was found to be higher in the control group (P<0.001). It was also observed that total protein (TP) (6.6±0.5 vs. 7.8±0.1 g/dL) (P<0.05) and Fb (914.3±68.6 vs. 265.4±19.8 mg/dL) (P<0.001) concentrations were higher in the TRP group, compared to the control group, while albumin (ALB) (1.9±0.2 vs. 3.1±0.1 g/dL) and Fe (47.00±5.29 vs.106.79±9.44 μg/dL) concentrations were significantly lower than the control group (P<0.001). In addition, a positive correlation was found between NO and Fb concentrations and between erythrocyte ARG activity and Fe concentrations. As a result, it was determined that NO concentrations were increased and erythrocyte ARG activity was not significant in dairy cows with TRP. In addition, increased plasma Fb concentration and decreased serum Fe concentration were determined in dairy cows with TRP. This study demonstrated that plasma NO, Fb and serum Fe concentrations in dairy cows with TRP may be useful markers for prognosis.

***Keywords:*** Arginase, dairy cows, iron, nitric oxide, traumatic reticuloperitonitis

*Corresponding Author:*
Akin Kirbas, Ataturk University, Faculty of Veterinary Medicine,
Department of Internal Medicine, 25240, Erzurum, Turkey
Email address: akindahiliye55@yahoo.com; akirbas@atauni.edu.tr

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## INTRODUCTION

Traumatic reticuloperitonitis (TRP) is a common disease in adult cattle caused by the ingestion and migration of foreign bodies in the reticulum. Perforation of the wall of the reticulum allows leakage of ingesta and bacteria, which contaminate the peritoneal cavity, resulting in local or diffuse peritonitis (Constable 2010; Constable et al., 2017). Especially pica occurring as a result of malnutrition is a disease risk factor for TRP (Ocal et al., 2008). These foreign bodies, such as nails or pieces of wire, perforate the wall of the reticulum and cause various complications, including reticulitis, peritonitis, pericarditis, pleuritis, hepatitis, and septicaemia (Ward and Ducharme 1994; Constable 2010; Braun et al., 2018). The clinical signs of cattle with TRP are variable, depending on the severity, duration, and involvement of other organs. Fever, increased heart and respiratory rates, anorexia, dehydration, decreased milk production, weight loss, ruminal atony, tympani, abdominal tension, abdominal pain and grunting are the most common clinical signs observed in cattle with TRP (Ward and Ducharme 1994; Constable 2010; Constable et al., 2017).

Nitric oxide (NO) is released from a variety of cells. It is generated from the terminal guanidine nitrogen atom of L-arginine by NO synthase (Marletta 1989; Gokce and Woldehiwet 2002). NO is an important molecule involved in physiological and pathological processes in animals. It can be protective or hazardous for organs or tissues in which it is present in biological fluids (Zelnickova et al., 2008). It has been reported that NO has pro-inflammatory and injurious effects on several systems (Van Der Vielt et al., 2000; Sharma et al., 2007). NO is known to play a major role in the primary defence against several species of bacteria (Degroote and Fang 1999; Nisbet et al., 2007; Hanedan et al., 2017), viruses (Issi et al., 2010; Kandemir et al., 2011) and parasites (Kontas and Salmanoglu 2006; Hanedan et al., 2015). NO also regulates the motility of the rumen and reticulum in cattle (Onaga et al., 2001). The activity of NO in cellular defence mechanisms includes participation in tissue injury and the mediation of inflammatory processes and apoptosis (Boucher et al., 1999; Wallace 2005).

Arginase (ARG) is the final enzyme of the urea cycle, and catalyze the hydrolysis of L-arginine to ornithine and urea. ARG has two isoforms. While ARG I is localized in the cytoplasm, ARG II is found in the mitochondria (Kepka-Lenhart et al., 2008). Although the urea cycle is present only in hepatocytes, the ARG enzyme is seen in many other cells. The liver has the highest content and it is active in the urea cycle to transform ammonia to non-toxic components (Spector et al., 1982; Fuentes et al., 1994). It has been reported to be present serves special functions, such as polyamine synthesis and the production of the proline required for protein biosynthesis, in addition to its functions in the urea cycle (Ozcelik and Ozdemir 2003).

Although there are studies on biochemical (Balikci and Gunay 2004; Bozukluhan and Gokce 2007a; Kirbas et al., 2015; Braun et al., 2018) and hematological parameters (Bozukluhan and Gokce 2007a; Kirbas et al., 2015; Braun et al., 2018), coagulation profile (Gokce et al., 2007), and some acute phase protein concentrations (Bozukluhan and Gokce 2007b; Kirbas et al., 2015) in cattle with TRP, there are not enough studies on erythrocyte ARG activity and plasma NO concentration. Therefore, in this study, we aimed to determine plasma NO concentration, erythrocyte ARG activity and fibrinogen (Fb) levels as well as serum iron (Fe) concentration and some biochemical parameters in TRP disease frequently encountered in dairy cattle.

## MATERIALS AND METHODS

**Animals.** 14 Swiss brown breed dairy cows with TRP referred to the Veterinary Teaching Hospital School of Veterinary Medicine, Firat University, were included in the study as an experimental group (TRP). 14 clinically healthy Swiss brown breed dairy cows were obtained from the dairy farm of the different region as a control group (CG). The animals in the TRP and CG consisted of cows in the lactation period and have given birth 4 to 6 times on average. All cows were adult ageing 4 to 8 years old. This study was conducted in accordance with ethical rules.

**Clinical examination and diagnosis.** The diagnosis of TRP was determined according to clinical, ferroscopy (Hauptner Ferroscope, Art-Nr 39500; H. Hauptner & Richard Herberholz GmbH & Co. KG, Solingen, Germany) and ultrasonographic findings and responses to pain tests. The CG consisted of cows with a negative response to these findings and tests. In the clinical examination of cows with TRP and healthy cows; rectal temperature (RT), heart (HR) and respiration (RR) rates and rumen contractions (RC) numbers were determined. It was detected that sick animals were brought to Veterinary Teaching Hospital

School of Veterinary Medicine, Firat University 1-2 days after clinical findings appeared. For the cows in the TRP group, slaughtering was recommended for those with Fb levels above 1000 mg/dl and conservative and platform treatments were recommended for those with Fb levels below 1000 mg/dl.

**Sampling.** Blood samples were taken only before treatment. Blood samples were taken from the jugular veins into vacuum tubes with anticoagulant (EDTA, 3.6 mg $K_2E$, Vacutainer, BD-Plymouth, UK) for plasma analyses and without anticoagulant tubes (Vacutainer, BD-Plymouth, UK) for serum analyses. Plasma and serum samples were separated by centrifugation at 3000 g for 10 minutes at room temperature and stored at -80°C until analyses. In addition, blood samples were also collected into vacuum tubes with heparin (Lithium heparin, Vacutainer, BD-Plymouth, UK) for the determination of ARG analysis.

**Biochemical assays**

**Plasma total NO.** A commercial NO detection kit (Enzo Life Science, Switzerland) was used for measuring plasma total NO level. The kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme nitrate reductase, followed by the colourimetric detection of nitrite as a coloured azo dye product of the Griess reaction that absorbs visible light at 540 nm.

**Erythrocyte ARG activity.** The erythrocyte ARG activity was determined using the thiosemicarbazide diacetyl-monoxime urea (TDMU) method (Geyer and Dabich 1971). The haemoglobin amount necessary for the determination of the erythrocyte ARG activity was ascertained with the Drabkin method depending on the cyanmethemoglobin formation (Drabkin and Austin 1932). In the present study, 1 unit of the enzyme was defined as the amount of enzyme generating 1 μmol urea from L-arginine in 1 hour at 37°C and stated as specific activity urea/hour/g haemoglobin.

**Plasma fibrinogen (Fb).** Plasma Fb concentrations were measured using the heat-precipitation method and were measured using a refractometer (Beijing, China) (Coles 1986).

**Serum biochemistry.** Serum enzyme activities alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), total protein (TP), and iron (Fe) concentrations were determined with commercial test kits by a biochemistry autoanalyzer (Beckman Coulter, AU5800, USA). The concentration of total globulin (GLOB) was calculated by subtracting the ALB concentration from the TP concentration (Roussel et al., 1997; Gokce et al., 2007).

**Statistical analysis**

Statistical analysis was performed using SPSS® (SPSS 16.0, Chicago, IL, USA) program package. Distribution of the data within groups was evaluated using a Shapiro-Wilk test. Parametrically distributed groups were compared using T-test (Independent-Samples T-Test). Levene's test was used to test whether variances were homogenous. Correlation between parameters was performed by Pearson Correlation test. Data were expressed as the mean ± standard error of the mean (SEM). The significance degree between two groups was determined to be $P<0.05$.

## RESULTS

**Clinical signs.** Mean values of clinical signs of dairy cows with TRP and CG were shown in Table 1. Body temperature ($P<0.05$), respiratory rate and heart rate of cows with TRP significantly increased compared to the control group ($P<0.001$) and the numbers of rumen contraction significantly decreased ($P<0.001$).

Additional findings of the cows with TRP were anorexia, grunting, constipation, tympani, ruminal stasis, impaction, abdominal pain and tension. Ferroscopy detected metallic foreign bodies with different response to the device (as 10-30 μA) around the reticulum and the cranio-ventral region of the rumen in the left side of the cows with TRP.

While there was a positive correlation between NO concentrations and heart and respiratory rates of TRP group, there was a negative correlation between NO concentrations and rumen contractions. In addition, there was a positive correlation between erythrocyte ARG activity and rumen contractions of the TRP group and a negative correlation between erythrocyte ARG activity and heart and respiratory rates (Table 4).

**Biochemical findings**

**ARG activity, NO, Fb and Fe concentrations.** The mean values of erythrocyte ARG activity, NO, Fb and Fe concentrations of dairy cows in TRP and CG were given in Table 2. In the TRP group, plasma NO (318.9±5.8/μmol/L-270.3±9.6/μmol/L) and Fb (914.3±68.6/mg/dL and 265.4±19.8/mg/dL) concentrations were higher than CG ($P<0.001$). However, in

the TRP group, the serum Fe concentrations (47.0±5.3/µg/dL and 106.8±9.4/µg/dL) and erythrocyte ARG activity (29.52± 0.5/Ug hemoglobin and 35.2±1.0/Ug hemoglobin) were lower than CG (P<0.001). In addition, in the TRP group, a positive correlation was found between plasma NO and Fb concentrations and between erythrocyte ARG activity and serum Fe concentrations. However, a negative correlation was determined between plasma NO concentrations and erythrocyte ARG activity (Table 4).

**Serum biochemistry.** The serum biochemical parameters of dairy cows with TRP and CG were shown in Table 3. Concentrations of TBIL, TP (P<0.05) and AST activity of TRP group were found higher than in CG (P<0.001), but concentrations of ALB of TRP group were determined lower than in CG (P<0.001).

**Table 1.** Mean values and standard error of the mean of clinical signs in dairy cows with TRP and control group.

| Parameters | Control group (n=14) | TRP group (n=14) | P value |
|---|---|---|---|
| RT (°C) | 38.5±0.1 | 39.0±0.1 | <0.05 |
| HR (beat/min) | 69.3±1.7 | 86.6±3.5 | <0.001 |
| RR (breaths/min) | 23.1±0.6 | 27.7±0.9 | <0.001 |
| RC (cyle/5 min) | 8.7±0.2 | 4.6±0.4 | <0.001 |

RT: rectal temperature; HR: heart rate; RR: respiration rate; RC: rumen contraction

**Table 2.** Mean values and standard error of the mean of erythrocyte ARG activity, NO, Fb and Fe concentrations in dairy cows with TRP and control group.

| Parameters | Control group (n=14) | TRP group (n=14) | P value |
|---|---|---|---|
| ARG (U/g haemoglobin) | 35.2±1.0 | 29.52±0.5 | <0.001 |
| NO (µmol/l) | 270.3±9.6 | 318.9±5.8 | <0.001 |
| Fb (mg/dl) | 265.4±19.8 | 914.3±68.6 | <0.001 |
| Fe (µg/dl) | 106.8±9.4 | 47.0±5.3 | <0.001 |

ARG: arginase; NO: nitric oxide; Fb: fibrinogen; Fe: iron.

**Table 3.** Mean values and standard error of the mean of serum biochemical parameters in dairy cows with TRP and control group.

| Parameters | Control group (n=14) | TRP group (n=14) | P value |
|---|---|---|---|
| AST (U/l) | 60.5±6.4 | 90.1±4.2 | <0.001 |
| ALP (U/l) | 58.0±9.6 | 96.8±27.7 | - |
| TBIL (mg/dl) | 0.2±0.0 | 0.4±0.1 | <0.05 |
| ALB (g/dl) | 3.1±0.1 | 1.9±0.2 | <0.001 |
| GLOB (g/dl) | 4.9±0.2 | 4.9±0.5 | - |
| TP (g/dl) | 7.8±0.1 | 6.6±0.5 | <0.05 |

AST:aspartate transaminase; ALP:alkhaline phosphatase; TBIL:total bilirubin; ALB: albumin; GLOB: globulin; TP: total protein; -: P>0.05.

**Table 4.** Correlation between NO, ARG, Fb, Fe, RT, HR, RR and RC in dairy cows with TRP.

| Parameters | NO | ARG | Fb | Fe | RT | HR | RR | RC |
|---|---|---|---|---|---|---|---|---|
| NO | 1 | -0.551** | 0.544** | -0.582** | 0.250 | 0.480** | 0.523** | -0.506** |
| ARG | | 1 | -0.569** | 0.640** | -0.218 | -0.489** | -0.462** | 0.649** |
| Fb | | | 1 | -0.586** | 0.320 | 0.582** | 0.504** | -0.827** |
| Fe | | | | 1 | -0.152 | -0.565** | -0.603** | 0.617** |
| RT | | | | | 1 | 0.506** | 0.252 | -0.441** |
| HR | | | | | | 1 | 0.549** | -0.550** |
| RR | | | | | | | 1 | -0.398** |
| RC | | | | | | | | 1 |

NO: nitric oxide; ARG: arginase; Fb: fibrinogen; Fe: iron; RT: rectal temperature; HR: heart rate; RR: respiration rate; RC: rumen contraction; **: P<0.01

## DISCUSSION

Traumatic reticuloperitonitis progresses in cattle as reticulitis, acute local and diffuse peritonitis, or chronic local and diffuse peritonitis. Besides, depending on contamination stages to surrounding organs, inflammation in reticulum and complications may also occur (Constable 2010; Constable et al., 2017). Progress of inflammation in the reticulum from acute process to chronic process makes treatment and healing process difficult. Therefore, the aim of this study was to determine plasma NO concentration, erythrocyte ARG activity and Fb levels as well as serum Fe concentration and some biochemical parameters in the dairy cows with TRP.

In the present study, a significantly different rectal temperature, respiratory and heart rates and rumen contractions were detected in the TRP group compared to the control group. It can be said that this situation may be due to the local or widespread inflammation in the reticulum region.

Nitric oxide is a signaling molecule that plays a key role in the pathogenesis of inflammation (Sharma et al., 2007). It involves in immune responses by cytokine-activated macrophages, which release NO in high concentrations (Wallace 2005). In consequence, large amounts of NO are synthesized, exceeding the physiological NO production by up to 1000-fold (Wallace 2005; Sharma et al., 2007). It was reported that NO concentrations in animals with bacterial (Nisbet et al., 2007; Li et al., 2010; Hanedan et al., 2017), viral (Kandemir et al., 2011; Bozukluhan et al., 2013) and parasitic diseases (Kontas and Salmanoglu 2006) increased compared to healthy controls. It was determined that NO concentrations increased in cattle with TRP (Atakisi et al., 2010) and traumatic pericarditis (Ozkan et al., 2012). The presence of pathogens, such as bacteria, and mucosal trauma resulting from TRP irritate the reticulum wall and stimulate mucosal NO production, thereby increasing NO synthesis (Yagmurca et al., 2009). The higher NO concentrations detected in the cows with TRP in the present study might be due to stimulation of the reticular mucosa by trauma caused by foreign bodies and possibly by entry of bacteria into the peritoneal cavity.

Arginase is a key enzyme of the urea cycle, an essential metabolic pathway for the removal of highly toxic ammonium ions resulting from protein degradation (Sharma et al., 2007). ARG activity is reduced by NO in the inflammatory process. It has been determined that ARG activity increases according to healthy animals in some viral (Issi et al., 2010; Kandemir et al., 2011), bacterial (Kandemir et al., 2013) and parasitic (Hanedan et al., 2015) diseases of cattle. In the present study was determined that ARG activity of TRP group was significantly lower than control group (Table 2). These findings supported the hypothesis that increased NO concentration in the inflammatory process decreased ARG activity.

Fb is one of the acute phase proteins (APPs) used to evaluate the inflammatory process in cattle (Cole et al., 1997; Jones and Allison 2007). Fb has been used for many years in inflammatory and traumatic diseases. It is characterized by a significant increase in response to trauma and infection. Plasma Fb concentrations in cattle increase within two days after trauma, inflammation and infection (Cole et al., 1997; Hirvonen and Pyörala 1998; Jones and Allison 2007). Hirvonen and Pyörälä (1998) have stated that Fb is useful for distinguishing TRP from other gastrointestinal diseases and pre-determination of the healing process of abdominal disorders. Gokce et al. (2007) stated that TRP is indicative of hyperfibrinogenemia. Therefore, Fb concentration is known to be useful for the diagnosis of TRP (Bozukluhan and Gokce 2007b; Kirbas et al., 2015). Kirbas et al. (2015) stated the increase in the Fb concentration was associated with the severity of inflammation process. Similarly, in this study, the Fb concentration of cows in the TRP group was significantly higher than in the control group (Table 2).

It was reported that Fe deficiencies were triggered by cytokines in the time of the inflammatory response. It is stated that Fe concentrations decrease during the acute phase response (APR) in the organism due to inflammation in horses (Borges et al., 2007), dogs (Torrente et al., 2015), adult cattle (Baydar and Dabak 2014) and calves (Aydogdu et al., 2018). Baydar and Dabak (2014) stated that serum Fe concentration in cattle with mastitis and TRP is significantly decreased compared to the control group and serum Fe concentration may be a useful parameter for the determination of inflammation. Borges et al. (2007) reported that the decrease in serum Fe concentration in horses is a sensitive marker of acute, subacute and chronic systemic inflammation, and the change in Fe concentration may be a useful parameter for monitoring response to treatment. Torrente et al. (2015) stated that serum Fe concentrations might also be a useful marker for the detection of acute inflammation in dogs with systemic inflammatory response syndrome (SIRS).

In a recent study was indicated that serum Fe concentrations were significantly reduced in calves with SIRS compared to the control group, and serum Fe concentrations could be a useful parameter for the determination of inflammatory response in calves with SIRS (Aydogdu et al., 2018). Similarly, in the present study, was determined that the Fe concentrations of the TRP group were significantly lower than that of control group (Table 2). Thus, it was determined that Fe could be a useful marker in the monitoring of the inflammatory process in cows with TRP.

Changes in TP, GLOB and ALB concentrations were expected in response to inflammation during the clinical form of TRP. In previous studies, TP concentrations have been determined as normal (Ozdemir 1989; Balikci and Gunay 2004; Kirbas et al., 2015; Braun et al., 2018), low (Batmaz 1990; Braun et al., 2018) or high (Ok and Aslan 1994; Gokce et al., 2007; Bozukluhan and Gokce 2007a; Braun et al., 2018) under these circumstances. In this study, TP concentrations of TRP group were low. Reticular abscess associated with TRP have been found to result in hyperglobulinemia (Balikci and Gunay 2004). Ok and Aslan (1994) stated that total globulin concentrations decreased during the disease as a result of protein migration into the inflammatory area. In this present study, mean GLOB concentrations in the TRP group were not found to be statistically different than the control group. The decrease in ALB may be linked to the synthesis of APPs (Kirbas et al., 2015), starvation, malnutrition and/or digestive failure (Balikci and Gunay 2004; Bozukluhan and Gokce 2007a). In this study, mean ALB concentrations in TRP group were different from control group. This result may reflect that hepatic ALB synthesis was affected by APR synthesis. Statistically significant differences in serum AST activity and TBIL concentration of TRP group compared to control group were detected (Table 3). These findings could be indicate that hepatocyte integrity of the liver was impaired in the cows with TRP.

In conclussion, it was determined that NO concentrations were increased and erythrocyte ARG activity was not significant in dairy cows with TRP. In addition, increased plasma Fb concentration and decreased serum Fe concentration were determined in dairy cows with TRP. This study demonstrated that plasma NO, Fb and serum Fe concentrations in dairy cows with TRP may be useful markers for prognosis.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Effects of Indigenous Spore-Forming Probiotic as Feed Supplement on Performance and Safety in Broilers

**N.G. Hosseini[1], M.H. Modarressi[1], S.N. Mousavi[2], M.T. Ebrahimi[3*]**

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Departments of Animal Science, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran*

[3] *Department of Biology, Faculty of Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran*

**ABSTRACT.** Probiotics colonize the intestine of animals and birds and provide useful effects on their performance and immune status. This study describes a high throughput screening and characterization of spore-forming bacteria from Iranian poultry farms with the aim to identify potential probiotic native *Bacillus* spp. and determine its effects on growth performance, hemato-biochemical parameters, immunity, intestinal microflora, morphology and *MUC2* gene expression of broiler chickens. A total of 300 one-day-old female Ross 308 broilers (42.6 ± 0.6 g) were used in a 6-wk study. Broilers were randomly allotted to 1 of 3 dietary treatments consisting of 4 replicate cages with 25 broilers each: 1- Control (Corn-soy-based diet: C), 2- C + 200 g/ton of the GalliPro® (*Bacillus subtilis* DSM 17299, $4\times10^9$ CFU/g, as positive control group: PC), 3- C + 200 g/ton of the native probiotic (*B. tequilensis K03,* $4\times10^9$ CFU/g: NP) identified in this study. During the experiment parameters were measured weekly. The results revealed that birds of the NP and PC groups exhibited improved feed conversion ratio (FCR) and increased body weight (BW), carcass and breast meat yield compared with the birds of the C group ($P<0.05$). Also, lymphocytes level, antibody titers against Newcastle diseases virus (NDV) and infectious bronchitis virus (IBV) of vaccinated birds were increased, while serum triglycerides, total cholesterol levels and abdominal fat of birds fed NP and PC were decreased compared to birds of the C group ($P<0.05$). The villus height, the relative expression of *MUC2* gene and *Bacillus* spp. populations were increased, while *E. coli* was significantly decreased in the ileum content of treated groups ($P<0.05$). These results indicate that the identified native *B.tequilensis* K03 strain can improve immunity and broiler performance by modifying intestinal microflora and morphology. Studied native probiotic *Bacillus tequilensis* K03 has useful effects on health status and it can be used as poultry feed supplement.

***Keywords:*** Broiler, Probiotic, *Bacillus*, Performance, Mucin

*Corresponding Author:*
Maryam Tajabadi Ebrahimi, Department of Biology, Faculty of Sciences, Central Tehran Branch, Islamic Azad University, Tehran, Iran
Email address: m.tajabadi@iauctb.ac.ir

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## INTRODUCTION

Antibiotics have been used in commercial poultry diet due to their growth-promoting and prophylactic effects for over 50 years (Coates *et al.,* 1963). Antibiotic intake of food animals, as well as the resulted antibiotic residue in food, has been noticed as one leading cause of the rapid spread of antimicrobial resistance in human populations. Reducing antibiotics in animal agriculture is one key in struggle against the spread of antibiotic resistance (Ghadban, 2002; Kabir, 2009). Increasing information on healthy food has led to increasing interests on natural food products such as probiotics. Probiotics have been demonstrated to improve intestinal microbial balance, provide protection against gut pathogens and modulate immune system. (Khaksefidi and Ghoorchi, 2006; Mingmongkolchai and Panbangred, 2018). These products have been identified as a safe feed additive in animal industry (Nawab *et al*., 2019). Lactic acid bacteria (LAB), mainly from genus *Lactobacillus*, consist the most important microbial population in the intestine of broiler chickens that have been used as probiotics in poultry industry (Huang *et al.,* 2004). Encapsulation technologies are used to keep probiotic cell viable all over storage, commercialization and use in food products, so that these cells are active during their passage through the gastrointestinal tract (Tellez *et al.,* 2012). *Bacillus* spp. is a genus of Gram-positive, rod shaped, and spore-forming bacteria. The spores present in vegetative cells allow long-term storage, and survival at the harsh environmental and processing condition and low pH of the gastrointestinal tract (Cutting, 2011).

The *Bacillus spp.* have been known as probiotics for chickens feed because it secretes antimicrobials compounds and suppress the colonization of gut pathogens (Hong *et al*., 2008; Knap *et al.,* 2011; Guyard-Nicodeme *et al.,* 2016). This probiotic with improve immunity (Melegy *et al.,* 2011) and changes in the intestine morphology of broilers (Sen *et al.,* 2012) lead to promote growth (Melegy *et al.,* 2011) and improves the quality of meat (Xu *et al.,* 2006; Yang *et al.,* 2016). Also, reported that *Bacillus spp.* decrease $NH_3$ emission from poultry manure (Jeong and Kim, 2014). Nonetheless, a few of them such as *B. subtilis, B. cereus,* and *B. licheniformis* are currently used in poultry industry, and the probiotic potential of other *Bacillus* spp. has been less studied (Cutting, 2011; Mingmongkolchai and Panbangred, 2018). The potential and efficacy of probiotics depend on the bacterial species and host origin, as well as on the application levels (Mountzouris *et al.,* 2007; Amerah *et al.,* 2013). Moreover, the antibiotic resistance of *Bacillus* spp. is another matter of concern. Therefore, exploring native or new probiotic strains is important to obtain very efficient probiotics for chicken feed. There is little information about the probiotic potential of *Bacillus tequilensis*, which biochemically is quite similar to *B. subtilis*, and can be differentiated by lysine decarboxylase, positive arginine hydrolases, ornithine decarboxylase and acid production from rhamnose (Gatson *et al.,* 2006). It is reported that *Bacillus tequilensis* K03 have the highest attachment ability to intestinal epithelium cells and inhibits the growth of *Salmonella* Typhimurium (Ghorban Hosseini *et al*., 2019). Therefore, in the present study, we investigated the effects a selected native strain (*B. tequilensis K03*) on performance and carcass traits, hemato-biochemical parameters, immunity, and intestinal morphology, microflora and *MUC2* gene expression of broiler chickens.

## MATERIALS AND METHODS

### Bacterial isolation and characterization

Bacterial isolates were obtained from fecal samples (n=86) collected from poultry farms in Golestan province in the north-east of Iran. The samples were serially diluted and spread plated on nutrient agar (QueLab-393506) followed by incubation at 37°C for 48 h. Discrete bacterial colonies (n=34) were picked and characterized according to Wu *et al.,* (2011). Then, probiotic characteristics (acid and antibiotic resistance, bile salt, the ability to attach to intestinal epithelial cells, and inhibit *Salmonella enterica* serovar Typhimurium invasion), as well as ability of producing amylase and phytase of *Bacillus* spp. isolates were analyzed (Latorre *et al.,* 2016; Thirabunyanon and Thongwittaya, 2012). The 16S ribosomal typing was also performed for identification of the selected strain (Jeevana Lakshmi *et al.,* 2013). All isolates were catalase-positive, oxidase-positive and non-hemolytic. The K03 strain was the superior bacterium, and had desirable probiotic characteristics, with production of 4.56 ± 1.1 U/ml phytase and 36.7 ± 1.3 U/ml α-amylase enzymes, and the highest adherence ability (1.9 log CFU/well) to intestinal epithelial cells. The strain had more inhibitory strength than the other isolates using exclusion assay to inhibit *Salmonella enterica* serovar Typhimurium attachment, up to 53% compared to control. The analysis of 16S rDNA gene sequences showed the highest similarity (% 99) of the K03 strain to *Bacillus tequilensis* KCTC 13622$^T$, in-

dicating the auto probiotic (indigenous bacteria) potential of the strain for use in chicken diet (Ghorban Hosseini *et al*., 2019).

**Birds and experimental design**

Three hundred one-day old healthy female broilers (Ross 308) with the initial weight of 42.6 ± 0.6 g were obtained from a local hatchery (Tehran, Iran), and randomly allocated to three dietary treatments (n=100) with four replicates (25 birds/pen) and raised for 42 days. The broiler chickens were fed a basal diet (Control; C) as well as basal diet + 200 g/ton of the GalliPro® commercial probiotic (*B. subtilis* DSM 17299, $4\times10^9$ CFU/g) as positive control group (PC). The birds in the native probiotic (NP) group were fed with basal diet + 200 g/ton of the native probiotic (*B. tequilensis K03,* $4\times10^9$ CFU/g) isolate. In broiler diets $8\times10^5$ viable spores/g was evaluated. Feed ingredients and nutrient composition of basal diet are shown in the Table 1. The environmental temperature was maintained at 32°C during the first week and gradually decreased (2°C per week) to 22°C, and then maintained constant until the end of the experiment. All guidelines for the ethical use and care of animal were followed, and approved by the Islamic Azad University Ethics Committee for Animal Experimentation.

**Table1.** Feed ingredients and nutrient composition of basal diet

| Ingredients (%) | Starter (1-10 d) | Grower (11-21d) | Finisher (22-42d) |
|---|---|---|---|
| Corn | 54.91 | 56.80 | 60.54 |
| Soybean meal (44% CP) | 38.00 | 36.22 | 32.03 |
| Soy oil | 2.51 | 3.00 | 3.83 |
| Limestone | 1.10 | 1.01 | 0.94 |
| Di-calcium phosphate | 1.93 | 1.65 | 1.42 |
| Vitamin and Mineral premix* | 0.50 | 0.50 | 0.50 |
| DL-methionine | 0.30 | 0.26 | 0.24 |
| L-lysine HCl | 0.25 | 0.13 | 0.10 |
| L-threonine | 0.11 | 0.06 | 0.03 |
| Common Salt | 0.20 | 0.23 | 0.24 |
| Sodium bicarbonate | 0.19 | 0.14 | 0.13 |
| Nutrient composition | | | |
| Metabolizable energy (kcal / kg) | 2950 | 3000 | 3100 |
| Crude protein (%) | 22.61 | 20.80 | 18.89 |
| Digestible lysine (%) | 1.26 | 1.11 | 0.99 |
| Digestible methionine + cysteine (%) | 0.93 | 0.84 | 0.77 |
| Digestible threonine (%) | 0.84 | 0.74 | 0.66 |
| Calcium (%) | 0.94 | 0.84 | 0.75 |
| Available Phosphorus (%) | 0.47 | 0.42 | 0.38 |
| Sodium (%) | 0.16 | 0.15 | 0.15 |

* The vitamin-mineral premix provided the following quantities per kg of diet: vitamin A, 9000 IU; vitamin D3, 2000 IU; vitamin E, 18 IU; vitamin K3, 2 mg; vitamin B1, 1.8 mg; vitamin B2, 6.6 mg; vitamin B3, 10 mg; vitamin B5, 30 mg; vitamin B6, 3 mg; vitamin B9, 1 mg; vitamin B12, 0.015 mg; biotin, 0.1 mg; choline chloride, 250 mg; antioxidant, 100 mg; Mn, 100 mg; Zn, 84.7 mg; Fe, 50 mg; Cu, 10 mg; I, 1 mg; Se, 0.2 mg.

**Growth performance**

Growth performance parameters, including body weight gain (BWG) and feed intake (FI) were measured while feed conversion ratio (FCR) was calculated for starter (1-10 d), grower (11-21 d), finisher (22-42 d), and overall period (1-42 days).

**Carcass yield and relative weight of organs**

On day 42, four birds from each replicates were randomly selected, weighed and slaughtered for carcass analysis, and determination of relative weight of organs. The weight of carcass, breast, thigh, gizzard, liver, heart, spleen and abdominal fat for each slaughtered bird was calculated as a relative percentage of live body weight (Zaghari *et al.,* 2016).

**Hematological parameters**

At the end of experiment, blood samples were collected from wing vein of four birds from each replicates, and divided into two aliquots. The first aliquot was transferred to a 2 ml heparinized tube containing EDTA to determine leukocytes, and other one in the same tube without anticoagulant and left to clot then serum was collected for humoral and biochemical analyses. Blood smears were prepared from each samples by Giemsa staining, and were examined un-

der a compound microscope for leukocyte differential count according to Beski and Al-Sardary (2015). Moreover, 100cells from the slides were evaluated to determine the heterophil to lymphocyte ratio.

**Serum biochemical analysis**

The collected blood samples (4 birds per replicate) in the 2 ml tube without anticoagulant left to clot, then serum was collected by centrifuging (1500 *g* for 15 min at 4°C), and stored at -20°C.

The concentration of serum total protein (TP), triglyceride (TG), glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were measured by commercial kits (Parsazmon Co. Iran) according to the manufacturer protocols, using the Eppendorf Auto analyzer (Epos5060).

**Humoral immune parameters (Antibody titers)**

The broilers were vaccinated against Newcastle, Influenza, and Infectious Bronchitis as described by Rahmani *et al*. (2005). On day 28 of the experiment, four birds from each replicate were bled by wing vein for serum antibody titer analysis (Rowghani *et al*., 2007). The samples were tested for Newcastle and Influenza disease by HI test (Xu *et al*., 1997), and were analyzed by an ELISA kit (Bronchitis, IDEXX Kit) for bronchitis diseases according to manufacturer's instruction.

**Determination of ileum microflora**

On day 42, four birds from each replicate were randomly selected and slaughtered, and then their ileum contents (1 g) were removed to determine microflora. The samples were diluted from $10^{-1}$ to $10^{-7}$ in normal saline solution, and then to determine *Bacillus* spp., *Lactobacillus* spp., and *Escherichia coli* counts, the diluted samples were seeded on Nutrient agar (QueLab-393506), MRS agar (Merck, Germany), and MacConkey agar (Merck, Germany), respectively, and incubated at 37°C for 48 h. The numbers of colony-forming units (CFUs) were expressed as log10 CFU per gram (Wu *et al.,* 2011).

**Ileum morphological examination**

At the end of experiment, 4 birds per replicate were sampled, and ileum (5 cm after Meckel's diverticulum) was taken, and fixed in 10% formalin. The 5 µm sections were prepared and stained with hematoxylin and eosin (H&E) for light microscopic (E600; Nikon) examination. Morphological experiments were performed according to Iji *et al.* (2001) methods, using Image-J software (http://rsb.info.nih.gov/ij/).

***MUC2* gene expression**

Total RNA was extracted from the 20 mg of the homogenized ileum intestinal samples (3 birds from each replicates) using the RNA Isolation Kit (QIAGEN, Cat. No. 74104), and then cDNA synthesis was performed in a total volume of 25 µl from 5 µl of extracted RNA using Quantifast Reverse-Transcriptase cDNA synthesis kit (QIAGEN, Cat. No. 205311) according to the manufacturer's recommendations. Relative expression of *MUC2* gene was quantified in duplicate for each cDNA sample on the Real-Time PCR detection system (Applied Biosystems) using Quanti Fast Syber Green PCR kit (QIAGEN, Cat. No. 204052), and specific primer pairs (BX930545; F: 5´-ATGCGATGTTAACACAGGACTC-3′; R:5´-GTGGAGCACAGCAGACTTTG -3´) with cycling parameters of 95°C for 10 min for 1 cycle, 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s for 40 cycles, as described previously by Forder *et al*. (2012). The melting curve of each amplicon was examined, and the expression of the *MUC2* gene was corrected based on the endogenous control expression (*GAPDH* gene: NM_204305; F: 5´-TGTGACTTCAATGGTGACAGC-3´, R: 5´-GCTATATCCAAACTCATTGTCATACC-3´) and calculated as fold change according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

**Statistical analysis**

The data were analyzed using the statistical package SAS software (SAS Institute, 2007) by one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test. Values of $P<0.05$ were considered statistically significant.

## RESULTS

**Growth performance**

The results of growth performance of broilers fed with diets containing native strain (*B. tequilensis* K03) and commercial product (*B. subtilis* DSM 17299) of *Bacillus* spp. probiotic are presented in Table 2. The results showed that dietary supplementation of *Bacillus* spp. probiotics (both native strain and commercial product) significantly improved BW, FCR of birds compared to the control group ($P<0.05$) during the overall period, while there was no significant difference ($P>0.05$) for starter, grower, and finisher periods. Feed intake was not affected by treatments. No significant differences were found in growth performance parameters between birds fed native strain and commercial *Bacillus* spp. probiotic supplemented diets ($P>0.05$).

**Table 2.** Growth performance of broiler chickens fed *Bacillus* spp. probiotics at different periods of experiment

| Parameters | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| ***1 – 10 days*** | | | | | |
| BW (g) | 238.3 | 243.7 | 244.8 | 2.0 | 0.4 |
| FI (g) | 266.2 | 235.2 | 233.8 | 11.3 | 0.4 |
| FCR | 1.1 | 0.9 | 0.9 | 0.05 | 0.3 |
| 11 – 21 days | | | | | |
| BW (g) | 601.4 | 625.9 | 627.5 | 7.5 | 0.3 |
| FI (g) | 852.1 | 835.2 | 834.4 | 16.3 | 0.9 |
| FCR | 1.4 | 1.33 | 1.3 | 0.02 | 0.2 |
| ***22 – 42 days*** | | | | | |
| BW (g) | 1720.6 | 1763.7 | 1762.5 | 10.1 | 0.1 |
| FI (g) | 3676.9 | 3663.8 | 3662.1 | 19.0 | 0.9 |
| FCR | 2.1 | 2.0 | 2.0 | 0.01 | 0.06 |
| ***1 – 42 days*** | | | | | |
| BW (g) | 2602.3 | 2675.4 | 2676.9 | 15.3 | 0.05 |
| FI (g) | 4795.3 | 4734.3 | 4730.4 | 42.8 | 0.8 |
| FCR | 1.8 [a] | 1.7 [b] | 1.7 [b] | 0.01 | 0.04 |

Different letters indicate significant differences between groups at $P<0.05$.
BW, Body weight; FI, Feed intake; FCR, Feed conversion ratio
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

**Carcass yield and relative weight of organs**

The results of carcass yield and relative weight of organs of broiler chickens are shown in Table 3. The relative weight of carcass, breast, thigh, and spleen were significantly increased and abdominal fat was decreased ($P\leq0.05$) in the birds fed with diets supplemented with *Bacillus* spp. probiotics (native strain and commercial product) as compared to the control during the overall experimental period. However, dietary *Bacillus spp*. probiotics had no significant effects on relative weight of liver, gizzard, and heart of the birds ($P>0.05$). No significant differences in carcass yield and relative weight of organs were observed between birds fed dietary native strain and commercial *Bacillus* spp. probiotics ($P>0.05$).

**Table 3.** Carcass yield and relative organ weight in broiler chickens fed *Bacillus spp.* probiotic diets at 42 d of age

| Parameters (%) | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| Carcass yield | 66.2 [a] | 67.3 [b] | 67.4 [b] | 0.2 | 0.05 |
| Breast | 22.4 [a] | 23.3 [b] | 23.4 [b] | 0.1 | 0.05 |
| Thigh | 16.1 [a] | 16.8 [b] | 16.8 [b] | 0.1 | 0.04 |
| Liver | 1.6 | 1.6 | 1.8 | 0.03 | 0.11 |
| Gizzard | 1.6 | 1.7 | 1.8 | 0.03 | 0.17 |
| Spleen | 0.1 [a] | 0.2 [b] | 0.2[b] | 0.01 | 0.04 |
| Heart | 0.8 | 0.9 | 0.8 | 0.07 | 0.96 |
| Abdominal fat | 1.6[a] | 1.4 [b] | 1.3 [b] | 0.04 | 0.04 |

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

**Hematological parameters (leukocytes)**

The effects of dietary supplementation of native strain and commercial *Bacillus* spp. probiotics on leukocytes differential count of broiler chickens are shown in Table 4. Diets containing native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299, respectively) significantly increased the percentage of lymphocytes compared to the control group ($P<0.05$), however, no significant differences were found between K03 and DSM 17299 groups ($P>0.05$). There was no significant differences in the percentage of heterophile, eosinophil, basophile, monocyte, as well as heterophile/lymphocytes ratio of birds fed diets containing native strain and commercial *Bacillus* spp. probiotics compared to the control group ($P>0.05$).

**Table 4.** Hematological parameters of broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age

| Parameters (%) | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| Heterophile | 31.6 | 31.5 | 31.3 | 0.3 | 0.9 |
| Lymphocytes | 51.8 [a] | 53.4 [b] | 53.9 [b] | 0.3 | 0.02 |
| Monocyte | 7.7 | 7.2 | 7.3 | 0.1 | 0.52 |
| Eosinophil | 2.6 | 2.7 | 2.7 | 0.05 | 0.74 |
| Basophile | 2.5 | 2.5 | 2.6 | 0.03 | 0.69 |
| Heterophile/Lymphocytes | 0.6 | 0.5 | 0.5 | 0.01 | 0.56 |

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

**Serum biochemical parameters**

The results of serum biochemical analysis of broilers fed with diets containing probiotic are shown in Table 5. The results revealed significant decrease in serum triglycerides and total cholesterol levels of birds fed dietary native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299) compared to the control group (P<0.05), however, no significant differences were found between K03 and DSM 17299 dietary groups (P>0.05). No significant differences were also found in serum glucose, total protein, High density lipoprotein (HDL), and Low density lipoprotein (LDL) levels among treatments (P>0.05).

**Table 5.** Serum biochemical parameters in broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age.

| Parameters | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| Glucose (mg $dl^{-1}$) | 261.0 | 235.9 | 246.9 | 12.6 | 0.75 |
| Total protein (g $dl^{-1}$) | 3.2 | 3.6 | 3.4 | 0.1 | 0.74 |
| Triglycerides (mg $dl^{-1}$) | 79.5 [a] | 66.9 [b] | 67.9 [b] | 2.2 | 0.02 |
| Total Cholesterol (mg $dl^{-1}$) | 163.2 [a] | 147.9 [b] | 149.3 [b] | 2.7 | 0.01 |
| HDL (mg $dl^{-1}$) | 65.3 | 59.8 | 62.4 | 1.7 | 0.49 |
| LDL (mg $dl^{-1}$) | 54.2 | 49.6 | 52.2 | 2.3 | 0.76 |

Different letters indicate significant differences between groups at $P<0.05$
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

**Humoral immune parameters (Antibody titers)**

The results of humoral immune responses of birds are shown in Table 6. Results revealed a significant increase (P<0.05) in antibody titers against Newcastle diseases virus (NDV) and infectious bronchitis virus (IBV) of vaccinated birds fed with diets containing native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299) in comparison with the control group (P<0.05), however, no significant differences were seen between K03 and DSM 17299 dietary groups. Moreover, diets containing *Bacillus spp*. probiotics had no significant effects on antibody titer against Influenza.

**Table 6.** Effect of *Bacillus* spp. Probiotics on immune response (antibody body production) of broiler chickens at 28 d of age

| Parameters | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| Bronchitis | 2676.2 [a] | 2772.7 [b] | 2784.5 [b] | 18.88 | 0.01 |
| Newcastle | 3.6 [a] | 4.6 [b] | 4.6 [b] | 0.19 | 0.03 |
| Influenza | 1.3 | 1.3 | 1.4 | 0.01 | 0.12 |

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

**Ileum microflora**

The effect of treatments on ileum microflora of broilers (42 d) is shown in Table 7. The results revealed that the native strain and the commercial *Bacillus* spp. probiotics (K03 and DSM 17299) significantly increased the *Bacillus spp.* Populations. *E. coli* was significantly decreased in the ileum content of birds fed with diets supplemented with probiotics as compared to control (P<0.05), however, no significant differences were found between the treated groups (P>0.05). Despite the slight increase in *Lactobacillus spp.* there were no significant differences between treated and control groups.

**Table 7.** Ileum bacterial counts [log (cfu/g)] of broiler chickens fed *Bacillus* spp. probiotic diets at 42 d of age

| Parameters | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| *Lactobacillus* spp. | 6.6 | 7.3 | 7.4 | 0.1 | 0.09 |
| *Bacillus* spp. | 5.6 [a] | 6.1 [b] | 6.3 [b] | 0.1 | 0.03 |
| *Escherichia coli* | 7.0[a] | 6.2 [b] | 6.1 [b] | 0.1 | 0.04 |

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

## Ileum morphology

The result of morphological analysis of ileum is shown in Table 8. No histopathological changes were observed in the intestine tissue of any birds of all feeding groups (Fig. 1). Morphological analysis of ileum revealed significant increases (P<0.05) in the villus height in birds fed with dietary containing *Bacillus* spp. probiotics (Native strain and commercial product) as compared to the control group, however, no significant differences were found between native and commercial probiotic dietary groups (P>0.05). There were no significant differences in the villus width, crypt depth, as well as villus height/crypt of ileum between experimental and control groups (P>0.05).

**Table 8.** Ileum morphology of broiler chickens fed *Bacillus* spp. probiotic diets at 42 d of age

| Parameters (μm) | C | PC | NP | SEM | P-value |
|---|---|---|---|---|---|
| Villus height | 769.0 [a] | 859.3 [b] | 889.7 [b] | 17.8 | 0.01 |
| Villus width | 153.6 | 154.2 | 165.2 | 4.7 | 0.53 |
| Villus height/crypt | 6.0 | 6.7 | 7.0 | 0.2 | 0.17 |
| Crypt depth | 133.1 | 134.5 | 132.6 | 3.9 | 0.97 |

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

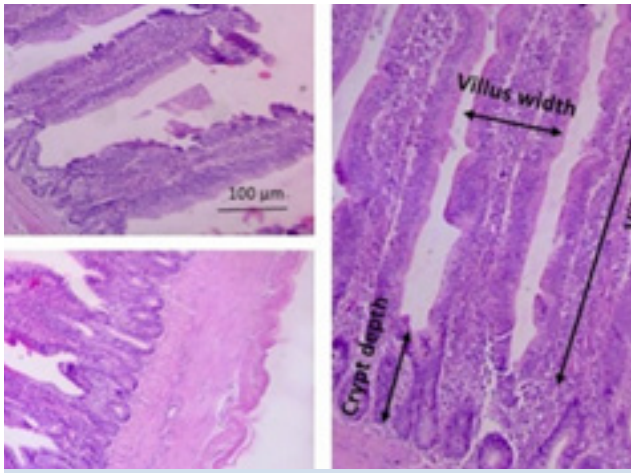


**Fig. 1.** Histological section (H&E) showing ileum morphology (Villus height, Crypt depth, and Villus width) of broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age. C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); SEM, Standard error of means.

## Intestinal *MUC2* gene expression

The effects of probiotic treated diets on expression of the intestinal *MUC2* gene are shown in the Fig. 2. The expression of intestinal *MUC2* gene was quantified by qPCR assay, and expressed relative to expression of the *GAPDH* gene. The relative expression of *MUC2* gene was significantly increased in the dietary native strain and commercial probiotics (K03 and DSM 17299, respectively) compared to the control group (P<0.05). No significant differences were found in *MUC2* gene expression between birds fed with K03 and DSM 17299 probiotic supplemented diets (P>0.05).

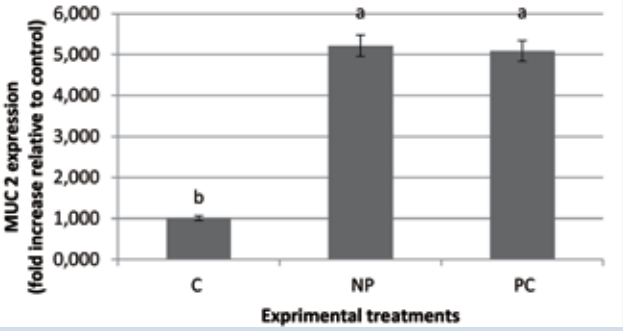


**Fig. 2.** The relative expression of *muc2* gene in the intestine tissue of broiler chickens fed *Bacillus* spp. probiotics diets at 42 d of age. C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, $4\times10^9$ CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, $4\times10^9$ CFU/g); Data were normalized based on endogenous *GAPDH* gene and presented as mean fold increase relative to the control ($2^{-\Delta\Delta ct}$ method). Different letters indicate significant differences between groups at $P<0.05$.

## DISCUSSION

The present study showed that consumption of diets supplemented with NP and PC significantly improved FCR and increased BW. The beneficial effects of dietary *Bacillus* spp. probiotic supplementation on FCR and increased BW of broilers are well documented in many studies (Opalinski *et al.,* 2007; Melegy *et al.,* 2011; Yang *et al*., 2016; Reis *et al.,* 2017). Spore-forming *Bacillus* spp. have been noticed as probiotic candidates due to their beneficial effects on animal health and growth, as well as their survivability under the harsh environment of the gastrointestinal tract, and stability during processing and long-term storage (Elshaghabee *et al*., 2017). Probiotics can modulate intestinal microflora, change intestinal morphology or secretion of enzymes and produce antimicrobial compounds. They can regulate immune system, increase the digestibility and the absorption of dietary nutrients and consequently improve the broiler performance (Ghadban, 2002; Elshaghabee *et al*., 2017). However, since the host origin microbes are quite familiar with the environment of gastrointestinal tract, the native and species-specific probiotic are highly preferred (Kabir, 2009). Similar studies showed that the improvement of broiler performance can be caused by beneficial changes of intestinal morphology and microflora (Ghadban, 2002; Elshaghabee *et al*., 2017). In this investigation increased BW and decreased FCR could be attributed to the growth of beneficial bacteria in the digestive tract, digestive enzymes production by these bacteria and improved digestion and absorption processes. The lack of impact in the initial period may be explained by the fact that probiotic bacteria are required to longer time for localization in the digestive tract. Our results showed that supplementation with the *B. tequilensis* K03 strain and commercial *B. subtilis* DSM 17299 have no effect on feed intake of chickens. Several studies (Opalinski *et al*., 2007; Melegy *et al*., 2011) have shown that feed intake of chickens was not affected by supplementation of *Bacillus* spp., suggesting that these strains cannot affect their appetite (Ferket and Gernat, 2006).

In our present study, increase in spleen relative weight, carcass, thigh and breast meat yield and decrease in abdominal fat of broiler chicks have been found when compared with the control group. These results are in agreement with those of Hatab *et al.* (2016), who reported that dietary supplementation with *Bacillus spp.* probiotics (*B. tequilensis* K03 strain and *B. subtilis* DSM 17299*)* rose carcass and relative organ weights due to increase of cell growth and turnover*,* while other researchers reported that (Afsharmanesh *et al*., 2014; Park *et al*., 2014; Reis *et al*., 2017; Shokryazdan *et al*., 2017), using the same or different probiotic species did not affect the relative organ weights of broilers. The reason for these contradictions may be due to differences in conditions of chickens, methods of administration, viability and concentrations of used bacteria, as well as the strain sources (Shokryazdan *et al*., 2017). Therefore, it seems probably that increase in carcass, thigh and breast meat yield of broiler in our present study can due to useful effect of probiotics in the growth of intestinal microbiota. In the report of Santoso *et al*. (2001) decrease synthesis and storage of fat in adipose tissue lead to decrease the percentage of abdominal fat.

Our results showed a significant increase in lymphocytes level, antibody titers against NDV and IBV of vaccinated birds. Lymphocytes play a crucial role in innate immune response, especially during stressful conditions, and participate in inflammation responses and phagocytosis. The increase in lymphocytes level indicates stimulation of the immune properties by *Bacillus* spp. probiotics that lead to increase in relative lymphoid organ weights (such as spleen). This assumption is supported by report of Neveling *et al*. (2017), who reported higher lymphocytes level in birds after dietary supplementation with probiotics.

It is strongly possible that probiotic microorganisms as an external organism stimulate the immune system, increase production the number of white blood cells and other immune compounds, the percentage of lymphocytes increased. Moreover, the ability of probiotics to promote humoral immunity in chickens vaccinated against Newcastle disease and infectious bronchitis reported by Rowghani *et al*. (2007), and in present study confirmed the immunostimulatory effects of the selected strain and the commercial *Bacillus* spp. probiotics. Probiotics control the balance of pro-inflammatory and anti-inflammatory cytokines. Cytokinins have an important role in immune responses. IFN-γ is a subset of the cytokine T-helper 1 that lead to killing organisms and protecting against all types of intracellular infections. Moreover interleukin-4 also can stimulate the differentiation of B cells and increase the production of antibodies to B cells (Belardelli, 1995). Therefore, the probable reason of increase in NDV and IBV of vaccinated birds is the stimulation of the immune system by probiotic native *Bacillus spp.* and probiotic Galpiro.

Our results showed a significant decrease in triglyceride and total cholesterol concentration in the serum of broilers fed with *B. tequilensis* K03 strain and *B. subtilis* DSM 17299 compared with the control group. Probiotics increase deconjucation of biliary acids excretion and since cholesterol is a substrate for the synthesis of bile acids, cholesterol molecules are used to produce bile acids (De Smet *et al*., 1998). Therefore they decrease the lipids level of blood.

In our present study, *Bacillus* spp. populations increased in the intestine of broilers fed the *B. tequilensis* K03 strain and *B. subtilis* DSM 17299. Several studies have demonstrated that dietary supplementation with *Bacillus* spp. modulate the microflora of broilers (Knap *et al*., 2011; Sen *et al*., 2012; Guyard-Nicodeme *et al*., 2016). Probiotics which increase the number of lactic acid bacteria in the gastrointestinal reduce its pH. Therefore, an unsuitable environment for the growth of harmful bacteria such as *E.coli* and *Salmonella* spp. is provided (Deniz1 *et al*., 2011). Therefore, it seems probably that probiotic native *Bacillus spp.* and probiotic Galpiro by pH reduction, increase beneficial bacteria and decrease *E. coli* population.

Our results indicated that the *B. tequilensis* K03 strain and *B. subtilis* DSM 17299 significantly increased villus height in ileum of the chickens. The effect of dietary *Bacillus* spp. probiotics on intestinal morphology of broilers has been well documented. Sen *et al.* (2012) reported the increased villus height and villus height to crypt depth ratio in chicken fed *Bacillus* spp. dietary. Deng *et al*. (2012) also found that dietary *Bacillus licheniformis* increased villus height in the ileum under heat stress conditions. It is showed that the digestive function of the intestine is related to villi structure and mucosal architecture, which influence absorptive capacity (Sen *et al.,* 2012; Neveling *et al*., 2017). Moreover, probiotics by short-chain organic acids formation stimulate the proliferation of epithelial cells and lead to increased villus height (Ichikawa *et al*., 1999).

The mucin secreted by goblet cells in the villi of the intestine is the main glycoprotein component of the mucus layer that it has role in modulation of intestinal microflora and health (Forder *et al*., 2007). In this study, intestinal *MUC2* gene expression under influence of two types of *Bacillus* spp. probiotic was significantly increased, suggesting that the probiotics may bind to specific receptor sites on the enterocyte and stimulate *MUC2* gene expression (Mattar *et al*., 2002).

## CONCLUSION

From this study, it can be concluded that the identified native *B. tequilensis* K03 strain can improve immunity, hemato-biochemical parameters, as well as broiler performance, which can be explained by the modified intestinal microflora, intestinal morphology changes and increase of *MUC2* gene expression. Since the effects of selected strain (*B. tequilensis* K03) were similar with the GalliPro® commercial probiotic (*Bacillus subtilis* DSM 17299) it can be used as probiotic potential for broilers feed.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Trans-diaphragmatic pressure measurement as a prognostic factor in the Intensive Care Unit in dogs

**K. Pavlidou[1,*], I. Savvas[1]**

[1]*Unit of Anaesthesiology and Intensive Care, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece*

**ABSTRACT.** In the last decade, attempts to improve the quality of the services provided to the critically ill patients in the Intensive Care Unit (ICU) are of great interest in human medicine. The aim of the majority of the clinical studies is the correlation of the survival rate of a critically ill patient with specific prognostic factors at the time of admission. The detailed assessment of a patient at admission in the ICU and during hospitalization seems to affect the management and the outcome. The main aim of this study was to evaluate if the trans-diaphragmatic pressure measurement can be a prognostic factor of the outcome in the ICU in dogs. Thirty-one dogs, 21 male and 10 female was included in this prospective, cohort study. Age, breed, sex, body weight and clinical diagnosis were recorded. The type of admission, the mentation status, physiological and biochemical parameters were measured at the admission of the dog in the ICU. All the variables were assessed over the first 24 hours following ICU admission. The animals were allocated into six groups: peritonitis/intra-abdominal surgery, intra-thoracic surgery, respiratory disease, neurologic disease, neoplasia, and systematic disease. The trans-diaphragmatic pressure ($P_{di}$) was measured under the same anesthetic level in all animals with two oesophageal balloon catheters. The most frequent problem for admission in ICU was peritonitis (5/31). Seventeen out of 31 were admitted in acute status while 14/31 had a chronic problem. Mean±standard deviation of $P_{di}$ was 10.7±5.6 mmHg and of lactate concentration 2.3±1.2 mmol/L. Both, they can predict outcome (p=0.071 and p=0.076, respectively). Seven out of 31 dogs died, 2 were euthanized and 22 were discharged from the ICU after hospitalization. The technique of $P_{di}$ measurement with balloon catheters can be successfully applied in dogs in the ICU. $P_{di}$ measurement, as well as lactate concentration may be used as prognostic indicators for the outcome, in dogs in the ICU. However, a bigger sample size is need to support these findings.

***Keywords:*** trans-diaphragmatic pressure, outcome, prediction, dog, ICU

*Corresponding Author:*
Kiriaki Pavlidou, 11 Stavrou Voutyra St, GR-546 27 Thessaloniki, Greece
E-mail address: kellypav@gmail.com

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## INTRODUCTION

In the last decade, attempts to improve the quality of the services provided to the critically ill patients in the Intensive Care Unit (ICU) are of great interest in human medicine (Kuzniewicz et al., 2008; Kvåle and Flaatten, 2002). The aim of the majority of the clinical studies is the correlation of the survival rate of a critically ill patient with specific prognostic factors at the time of admission. The detailed assessment of a patient at admission in the ICU and during hospitalization seems to affect the management and the outcome. In human medicine, the clinical condition of a patient in the ICU is assessed according to particular objective risk models. Scoring systems for illness severity are based on a number of clinical variables that predict mortality risk, and provide an objective basis for patient triage. These systems should be based on objective criteria, be accurate and easy to use (Gunning and Rowan, 1999). According to the human literature, the most used objective risk model that is applied in the ICU is the APACHE II (Acute Physiology and Chronic Health Evaluation System). The basis for the APACHE development was the hypothesis that the severity of acute disease can be measured by quantifying the degree of abnormality of multiple physiological parameters. In particular, the APACHE II is formed based on three parameters: the assessment of 12 physiological parameters (e.g. heart rate, respiratory rate, mean arterial pressure, temperature, oxygenation, arterial pH, hematocrit), the age of the patient and the presence of chronic surgical disease (Knaus et al., 1985; Niewiński, 2014).

The physiological status of the critical ill patients is characterized by rapid and frequent life-threatening alterations in organ function. The respiratory function is often affected during the hospitalization of a patient in the ICU. Respiratory muscle fatigue may develop, either in primary or secondary respiratory disease, or as a complication during the hospitalization. According to the literature, the majority of the cases in the ICU involve the respiratory system (Eng et al., 1992). Despite the evidence that respiratory muscle dysfunction develops in critically ill patients which contributes to weaning failure, the respiratory muscles are poorly monitored in the ICU (Hermans et al., 2010; Jaber et al., 2011; Laghi et al., 2003). Three factors may contribute to this: 1. the limited knowledge on the effects of critical illness on respiratory muscle function, 2. the lack of knowledge and availability of tools to monitor respiratory muscle function, and 3. the perception that the monitoring of respiratory function has no clinical consequences (Doorduin et al., 2013).

The diaphragm is the main respiratory muscle and it is thought to be very sensitive to respiratory muscle fatigue (Jonville et al., 2002; Moxham et al., 1981b, Moxhman et al., 1981a; Zakynthinos and Roussos, 2005). As respiratory muscle fatigue is defined the inability of the muscle to produce pressure in order to maintain the alveolar ventilation, and it is reversible during rest (Lumb, 2010; Zakynthinos and Roussos, 2005). The effects of critical illness on respiratory function are often part of a generalized phenomenon which is known as "ICU-acquired weakness". The main causes of this phenomenon may be systemic inflammation, drugs, electrolyte disturbances and immobility (Jolley and Bunnell, 2016). Additionally, the prolonged mechanical ventilation in the ICU patients leads to decreased diaphragmatic strength, known as "ventilator-induced diaphragmatic dysfunction" (Vassilakopoulos, 2012).

There are many methods to monitor the respiratory muscle function. Pressure and flow recordings, electromyography, ultrasonography, circulatory biomarkers, computed tomography (CT) and magnetic resonance imaging (MRI) (Doorduin et al., 2013). However, the best indicator of diaphragmatic contractility seems to be the trans-diaphragmatic pressure ($P_{di}$) measurement. Specifically, the measurement of $P_{di}$ during a maximum inspiratory effort ($P_{di\ max}$) is an indicator of the strength of the diaphragm and it helps to assess patients with respiratory muscle weakness (Chen et al., 2000; Ferguson, 1994; Man et al., 2004; Man et al., 2002).

$P_{di}$ is defined as the difference between the intra-abdominal pressure ($P_{abd}$) and the intra-pleural pressure ($P_{pl}$) (Adams et al., 1988; Gilbert et al., 1979; Hubmayr et al., 1990). However, the measurement of $P_{abd}$ and $P_{pl}$ is not straightforward under clinical conditions and therefore alternative, less invasive techniques have been proposed, replacing the measurement of $P_{abd}$ and $P_{pl}$ by the measurement of the intra-gastric pressure ($P_{gast}$) and the intra-oesophageal pressure ($P_{oes}$), respectively (Benditt, 2005). This approach has been adapted for use in dogs allowing the monitoring of $P_{di}$ in a clinical setting (Pavlidou et al., 2014). In experimental studies in humans and in animals, the maximum inspiratory pressure is achieved by the electrical stimulation of the phrenic nerves (Hubmayr et al., 1990; Leduc et al., 2008) However, non-invasive methods (Mueller's manoeuvre) have been used in co-operative patients for the measurement of $P_{di\ max}$.

With this method, the maximum $P_{gast}$, $P_{oes}$ and $P_{di}$ can be measured in one respiratory cycle (De Troyer and Estenne, 1981).

In human medicine, there is an effort to apply $P_{di}$ measurement in the ICU in a routine setting in order to assess the critical ill patients. Recent studies have shown that respiratory muscle fatigue can develop in mechanically ventilated patients in the ICU and this can result in respiratory failure (Demoule et al., 2013; Hermans et al., 2010; Supinski and Ann Callahan, 2013). The respiratory muscle weakness is the result of the absence of diaphragmatic activity, because of the application of mechanical ventilation, and it is worsened with the prolongation of the mechanical ventilation (Petrof, 2013). However, other factors such as hyperglycemia, azotemia and hypoalbuminemia can cause respiratory muscle weakness in critically ill patients in the ICU (Hermans et al., 2007; Modawal et al., 2002; Wu et al., 2009).

Recently, in veterinary clinical practice, the detailed assessment of the critical ill patients in the ICU has been studied. The APPLE (Acute Patient Physiologic and Laboratory Evaluation) score system is the risk model that can be used in the veterinary ICU. Physiological parameters (e.g. sex, age, weight), hematological and biochemical profile, chronic diseases, previous surgical procedures and therapeutic treatments are some of the factors that are assessed in the APPLE score system (Hayes et al., 2010b). Although, critically ill dogs are monitored carefully in the ICU, the evaluation of the respiratory function is not a routine procedure in a clinical setting in these patients. There are few available data for the measurement of $P_{di}$ in veterinary practice, as the most studies were "*in vitro*" and the diaphragmatic contractility was evaluated after the electrical stimulation of the phrenic nerves. However, there are two recent clinical studies in dogs where the modified Mueller's manoeuvre was applied for the $P_{di}$ measurement, with the placement of two oesophageal balloon catheters, one in the oesophagus and one into the stomach, and the effect of different anesthetic protocols on diaphragmatic contractility was studied (Pavlidou et al., 2014, Pavlidou et al., 2013).

To our knowledge, there is no clinical study in veterinary medicine on the measurement of $P_{di}$ as a prognostic factor for the outcome of a clinical case in critically ill dogs. The aim of this study was the evaluation of the $P_{di}$ measurement in animals admitted and hospitalized in the ICU as prognostic factor for the outcome, among other clinical measurements, in a clinical setting.

## MATERIALS AND METHODS

Approval from the Ethics Committee of the Aristotle University of Thessaloniki was obtained (2016-050-0503-8401). All the dog owners were informed in detail about the study protocol and a signed written consent was obtained. The study population was dogs admitted to the Intensive Care Unit of Companion Animal Clinic of Aristotle University. The animals were excluded from the study when the collection of the data was impossible (e.g. ineffective measurement of $P_{di}$). Another exclusion criterion was obesity which has been shown to decrease diaphragmatic contractility (De Keulenaer et al., 2009; Lambert et al., 2005; Ora et al., 2011).

Thirty-one client-owned dogs were enrolled in this observational prospective cohort study. In each dog, age, breed, sex, body weight, clinical diagnosis, and the acute or chronic onset of illness were recorded within the first 24 hours following ICU admission; mentation score was assessed at admission in order to estimate the true baseline mental status before the administration of any analgesia or sedation (Hayes et al., 2010a).

The primary system affected at admission was identified and the dogs were allocated to six groups: peritonitis/intra-abdominal surgery, intra-thoracic surgery, respiratory disease, neurologic disease, neoplasia and systematic disease.

Physiological and biochemical parameters were measured at the admission of the dog in the ICU. Full clinical examination and estimation of consciousness status were performed. The dogs were assigned to one of five levels of consciousness: normal mentation, depression, lethargy, coma and excitement (Hayes et al., 2010b). Heart rate (HR), auscultation of the thorax, electrocardiography (ECG), mucous membrane color, pulse quality and invasive arterial blood pressure (systolic/SAP, diastolic/DAP and mean/MAP) were measured (Mindray, iPMI 12 Vet, Shenzen Mindray Bio-Medical Electronics CO, LTD, Nanshan, China). Respiratory rate (RR) was measured by observation and capnography (Datex-Ohmeda S/5, GE Healthcare, Finland) and the end-tidal carbon dioxide concentration ($ETCO_2$) was estimated also by capnography. Oxyhemoglobin saturation ($O_2SAT$) was estimated indirectly by pulse oximetry (Mindray, iPMI 12 Vet, Shenzen Mindray Bio-Medical Electronics CO, LTD, Nanshan, China) and calculated from the oxygen par-

tial pressure measured by arterial blood gases analysis (Siemens RapidPoint 500, Siemens Healthcare Diagnostics, New York, USA). The laboratory parameters were hematocrit (HCT), white blood cells (WBC) and platelets (PLT) count, total solids (TS), urea, creatinine (Crea), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose (Glu) and electrolytes ($K^+$, $Na^+$, $Ca^{+2}$). Lactate concentration was also measured at the time of admission (Accutrend Plus System, Roche Hellas, Greece). The arterial blood gases analysis gave information about the pH, oxygen partial pressure ($PO_2$), carbon dioxide partial pressure ($PCO_2$), bicarbonate concentration ($HCO_3^-$), $O_2SAT$ and the ratio oxygen partial pressure/fraction of inspired oxygen ($PO_2/FiO_2$) (Siemens RapidPoint 500, Siemens Healthcare Diagnostics, New York, USA).

The trans-diaphragmatic pressure was measured under the same anesthetic level in all animals. When the anesthetic level was deep (lack of reflexes, adequate muscle relaxation, lack of response to surgical stimulation), two 90 cm long oesophageal balloon catheters with guide wires (Esophageal Balloon Catheter Set; Coopersurgical Company, CT, USA) were introduced orally. Using the landmarks that have been described (Pavlidou et al., 2014; Waterman and Hashim, 1991), the balloon of the first catheter was introduced into the stomach for the measurement of $P_{gast}$ and the distal end of the second catheter was positioned in the mid-third of the oesophagus for the measurement of $P_{oes}$. The correct positioning of the balloon catheters was confirmed by the observation of positive and negative pressure tracings of $P_{gast}$ and $P_{oes}$, respectively on a computer screen. The catheters were secured in place by fixing them on the endotracheal tube. The guide wires were removed, the catheters were connected to the pressure transducers and the balloons were inflated with 0.5-1 ml of air. The electrical connections from the transducers were attached to a pressure monitoring device with the appropriate software (Pressure Monitoring system Buzzer-II; Michael Roehrich, Austria) and then to a computer. The pressure transducers were zeroed to the atmospheric pressure prior to each measurement.

In order to obtain the maximum $P_{oes}$, $P_{gast}$ and $P_{di}$, a modified Mueller's manoeuvre was applied. Particularly, the endotracheal tube was disconnected from the anaesthetic circuit and the proximal end of the tube was tightly closed with a thumb during the respiratory pause after the end of expiration, and thus forcing the dog to breath against the obstructed airway (modified Mueller's manoeuvre (Pavlidou et al., 2014).

As the animals were critically ill patients, the anaesthetic protocol could not be the same for all of them. In each case, the anaesthetic protocol was based on the clinical condition of the animal and it was selected in a way to minimally affect the $P_{di}$ (Pavlidou et al., 2013). Specifically, the premedication differed among the animals, whereas the induction and the maintenance of anaesthesia was the same in all animals. Anaesthesia was induced with propofol (Propofol MCT/LCT, Fresenius, Fresenius Kabi, Greece) intravenously to effect. An initial dose 1-2 mg/kg was given followed, if needed, by incremental doses of 0.5-1 mg/kg until endotracheal intubation could be easily performed. Anaesthesia was maintained with isoflurane (Isoflurane, Merial, Italy) in oxygen. All animals were breathing spontaneously. Fresh gas (100% oxygen) flow was delivered at 1.5 L/min through a circle rebreathing system. The days of the hospitalization were recorded in all animals and the outcome was assessed as alive, dead or euthanized.

The recorded data of the $P_{di}$ were saved in as spreadsheet and analyzed with a signal analysis software (Qtiplot, MicroCal, Northampton, Massachussets, USA). A positive curve of the gastric pressure and a negative curve of the oesophageal pressure, were drawn. The baseline of gastric and oesophageal curves was zeroed and the $P_{di}$ value was calculated (difference between $P_{gast}$ and $P_{oes}$). Binary Logistic Regression Analysis was used to evaluate the effect of the predictive variable on the patient outcome. Predictive variables included in the model were group, problem, admission, mental status, lactate concentration, and $P_{di}$ value. The designed model was: log(p/1-p) = constant + b1 x problem + b2 x admission + b3 x mental status + b4 x lactate concentration + b5 x $P_{di}$., where p is the probability of a dog to be alive.

## RESULTS

Thirty-one dogs (21 male, 10 female), 1-15 (6.7±4) years (mean±standard deviation) old, weighing 3-40 kg (16.8±12.3) were included in the study. The most frequent problem for the hospitalization in the ICU was peritonitis (5/31), followed by brachycephalic upper airway syndrome, Wobbler syndrome and status epilepticus (Table 1). Seventeen out of 31 were admitted after an acute onset, while 14/31 had a chronic problem. Moreover, 51.6% were in normal mentation and 48.4% were in depression.

**Table 1.** The causes for admission in ICU

| Problem | Frequency |
|---|---|
| **peritonitis/intra-abdominal surgery** | **11** |
| ileus | 1 |
| laparotomy | 1 |
| peritonitis | 5 |
| pyometra | 1 |
| splenectomy | 1 |
| gastric dilation/volvulus | 2 |
| **intra-thoracic surgery** | **5** |
| pericardiectomy | 1 |
| patent ductus arteriosus | 2 |
| diaphragmatic hernia | 2 |
| **respiratory** | **5** |
| Brachycephalic upper airway syndrome | 3 |
| dyspnoea | 1 |
| laryngeal paralysis | 1 |
| **neurologic** | **6** |
| Wobbler syndrome | 3 |
| status epilepticus | 3 |
| **neoplasia** | **3** |
| lung tumor | 1 |
| other | 2 |
| **systematic** | **1** |
| sepsis | 1 |

Ten dogs were premedicated with dexmedetomidine (Dexdomitor, Pfizer, Greece) at 175 μg/m$^2$ intramuscularly (IM) alone or in combination with methadone (Synthadon, LeVet, The Nederlands) at 0.1 mg/kg IM, 5 dogs with acepromazine (Acepromazine, Alfasan, The Nederlands) at 0.05 mg/kg IM and methadone at 0.1 mg/kg IM, 12 dogs with fentanyl (Fentanyl, Jansseg-Cilag, Greece) at 1 μg/kg and midazolam (Dormipnol, Viofar, Greece) at 0.5 mg/kg intravenously, and finally 4 dogs were not premedicated at all.

Descriptive statistics for the hemodynamic, respiratory and biochemical parameters are shown in Table 2. $P_{di_mean}$ was 10.7±5.6 mmHg and the $PO_2/FiO_2$ ration was 348.5±145.4 mmHg. The lactate concentration was 2.3±1.2 mmol/L. The duration of hospitalization was 2±1.8 days. Seven dogs died, 2 were euthanized and 22 were discharged from the ICU after hospitalization. In this clinical study, 16/31 dogs had a normal ratio, ARDS was developed in 5 animals and ALI in one. Eleven out of 16 dogs were discharged, while 3/5 with ARDS and 1/1 with ALI died.

The binary regression analysis results are shown in Table 3. Lactate concentration and $P_{di}$ can both predict outcome with a good probability.

**Table 2.** Mean±standard deviation for the hemodynamic, respiratory and biochemical parameters

| Variable | Mean±standard deviation | Variable | Mean±standard deviation |
|---|---|---|---|
| HR (min$^{-1}$) | 122±27.3 | pH | 7.3±0.12 |
| MAP (mmHg) | 61±17.4 | $PO_2$ (mmHg) | 346.4±159.3 |
| RR (min$^{-1}$) | 15.3±6.4 | $PCO_2$ (mmHg) | 48.8±14.1 |
| HCT (%) | 36.3±8.1 | $HCO_3^-$ (mmHg) | 21.8±2.8 |
| GLU (mg/dL) | 105.7±18.8 | $ETCO_2$ (mmHg) | 46.5±11.5 |
| $K^+$(mmol) | 4±0.8 | $P_{di}$ (mmHg) | 10.7±5.6 |
| Lactate (mmol) | 2.3±1.2 | $PO_2/FiO_2$ | 348.5±145.4 |

**Table 3.** Binary logistic regression analysis for outcome with predictive variables in the model: group, problem, admission, mental status, lactate concentration, and $P_{di}$ value (b=coefficient, p=observed probability). The value "respiratory problem" has been defined as baseline value for the analysis.

| Variable | b | p | Odds ratio |
|---|---|---|---|
| admission | -3.640 | 0.149 | 0.026 |
| mental status | 0.215 | 0.863 | 1.240 |
| lactate | -0.935 | 0.071 | 0.393 |
| $P_{di}$ | -0.354 | 0.076 | 0.702 |
| problem | | 0.602 | |
| peritonitis/intra-abdominal surgery | 28.139 | 0.999 | $1.66 \times 10^{12}$ |
| intra-thoracic surgery | 26.284 | 0.999 | $2.59 \times 10^{11}$ |
| neurologic | 31.662 | 0.999 | $5.63 \times 10^{13}$ |
| neoplasia | 32.787 | 0.999 | $1.73 \times 10^{14}$ |
| systematic | 23.926 | 1.000 | $2.46 \times 10^{10}$ |
| constant | -19.343 | 1.000 | 0 |

## DISCUSSION

The aim of this clinical cohort study was the evaluation of the $P_{di}$ measurement in animals admitted and hospitalized in the ICU as prognostic factor for the outcome, among other clinical measurements, in a clinical setting. The evaluation of diaphragmatic contractility was based on $P_{di}$ measurement with balloon catheters. To the best of the authors' knowledge, this is the first application of the $P_{di}$ measurement in a non-fatigued diaphragm in dogs in the ICU. Diaphragmatic contractility and $P_{di}$ measurement with balloon catheters has been studied in fatigued, intact diaphragm after phrenic nerve stimulation in healthy (Araujo and Milic-Emili, 2005; Gilbert et al., 1979; Higgs et al., 1983) and critically ill human patients (Watson et al., 2001). The mean value of $P_{di}$ in critically ill patients after phrenic nerve stimulation has been reported to be 7.87 mmHg, (Watson et al., 2001) which is lower than the mean values in the present study.

In a study in dogs, (Pavlidou et al., 2014) the Mueller's manoeuvre, the most commonly used manoeuvre for the measurement of $P_{di}$ in human medicine, has been modified and applied for three consecutive respiratory cycles for the $P_{di}$ measurement. The same manoeuvre was applied in the present study. Post-obstructive pulmonary edema (POPE) may occur as a complication of the Mueller's manoeuvre. However, there is no reference to the development of POPE in dogs and in awake humans in a clinical setting (Pavlidou et al., 2014). In our clinical study, clinical signs of POPE have not been observed in any animal.

The premedication could not have been the same in all the critically ill patients, because of their different clinical situation. This is a limitation of the study, as the different premedications may have variably affected the diaphragmatic contractility. However, the protocol for the induction and the maintenance of anaesthesia was the same in all animals. According to a previous study, fentanyl and propofol seem to reduce diaphragmatic contractility, as $P_{di}$ values were 12.0±5.9 mmHg and 12.2±3.2 mmHg respectively, in comparison with isoflurane (14.9±4.7 mmHg) in dogs under anaesthesia (Pavlidou et al., 2013).

Lactate concentration is considered to be a useful tool in human and veterinary clinical practice. Hyperlactatemia and lactic acidosis occur frequently in veterinary ICU patients with shock, low cardiac output, acute liver failure, sepsis, neoplasia, peritonitis, poisoning and drug therapy (de Papp et al., 1999; Lagutchik et al., 1998, Lagutchik et al.,1996). In healthy adult dogs at rest, lactate concentration is <2.0 mmol/L, but it may be measured as high as 3.5 mmol/L (Lagutchik et al., 1996). In humans, many studies have estimated the prognostic values of lactate concentration levels. It has been shown that a single measurement of lactate concentration is associated with the prognosis of survival (Bernardin, 1996; Cerović et al., 2003). In veterinary medicine, there seems to be a relationship between lactate concentration and outcome in dogs. According to Lagutchik et al., dogs admitted to the ICU with various underlying diseases, having high blood lactate levels at admission were more likely to die (Lagutchik et al., 1998). Animals with high blood lactate levels (>4-5 mmol/L) have a very poor prognosis after 24 hours of the admission. Gastric dilation/volvulus and peritonitis are two pathological conditions where lactate concentration is considered to be a good prognostic factor in dogs (Cortellini et al., 2015; de Papp et al., 1999).

In the present study, lactate concentration along

with $P_{di}$ measurement, may predict outcome, with low p values (0.071 and 0.076, respectively). The combination of these two measurements at admission may be of clinically good prognostic value, in dogs and in an animal hospital setting.

Recently, the study of the $PO_2/FiO_2$ ratio has gained a lot of interest in human and veterinary medicine. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are two life-threatening syndromes with high morbidity and mortality (Matthay et al., 2012; Rubenfeld et al., 2005). According to the American European Consensus Conference (AECC),(Bernard et al., 1994) in humans, a mean value of $PO_2/FiO_2$ <300 mmHg indicates ALI and <200 mmHg ARDS. As there are no reference values for ARDS and ALI in dogs, the same normal values of $PO_2/FiO_2$ has been suggested for dogs (Calabro et al., 2013).

The cause of admission could be another prognostic factor for the outcome of the dogs in the ICU. Intra-abdominal infections are an important cause of ICU morbidity and mortality. Peritonitis develops as complication in 30% of the human patients with intra-abdominal infection in the ICU, increasing the mortality rates up to 50% (Delibegovic et al., 2011; Marshall and Innes, 2003). In veterinary medicine, peritonitis is a major problem in the ICU but there is no study to correlate this with the outcome of a case.

Limitations of the study include all those factors that affect the $P_{di}$ measurement. First of all, the $P_{di}$ measurement with balloon catheters is feasible only under general anaesthesia in dogs. Because of this, it cannot be applied to all animals in the ICU. Thus, in our study, the measurement of $P_{di}$ was not applicable in all cases. Moreover, the return of the diaphragmatic contractility back to its normal function after the hospitalization in the ICU is not always applicable, for the same reason. Another limitation of the study was that the insertion of the balloon catheter was impossible in some cases, because the catheter could not pass through the lower esophageal sphincter.

In summary, the technique of $P_{di}$ measurement with balloon catheters can be successfully applied in dogs in the ICU, although there are some limitations. $P_{di}$ measurement with lactate concentration at admission may also be good prognostic indicators for the outcome, although a larger sample size to support this is needed.

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## CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

# Distribution of serotypes of *Listeria monocytogenes* in chicken meats in Turkey

**S. Sahin[1], R. Kalin[2], MN. Mogulkoc[2]**

[1]*Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Sivas, Turkey.*

[2]*Department of Microbiology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Sivas, Turkey.*

**ABSTRACT.** *Listeria monocytogenes* is one of the important causes of food-borne infections. This study was conducted to determine the presence of *L. monocytogenes* and its serotype distribution in a total of 400 packaged chicken meat products (drumstick, breast, wing, and whole chicken) from different national companies. *L. monocytogenes* contamination was detected in 26.5% (106 in 400) of all samples when the products considered, drumsticks, breasts, wings, and whole chickens showed 47%, 15%, 35, and 9% positivity respectively. Four important serotypes of *L. monocytogenes* in human listeriosis (1/2a, 1/2b, 1/2c and 4b) were identified, and serotype 1/2a (94.3%) was determined as predominant in packaged chicken meats. The present study revealed that *L. monocytogenes* 1/2a serotype is prevalent in chicken meats and this may cause public health problems in Turkey. Further studies in poultry meats should be conducted on a large scale such as regional or national big markets to determine the presence of the pathogen and its dominant serotypes.

***Keywords:*** *Listeria monocytogenes;* food-borne pathogen; serotype 1/2a; mPCR, chicken meats.

*Corresponding Author:*
Seyda Sahin, Department of Food Hygiene and Technology,
Faculty of Veterinary Medicine, Sivas Cumhuriyet University, 58140 Sivas, Turkey.
Orcid ID: https://orcid.org/0000-0002-8173-7818
E-mail address: seydasahin@cumhuriyet.edu.tr

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## INTRODUCTION

As one of the important food-borne infectious agents, *Listeria monocytogenes* is still a serious concern for public health with its very high mortality rate (Farber and Peterkin, 1991; Oliveira et al. 2018). *L. monocytogenes* is a specific risk for the health of vulnerable people especially elderly people, immunocompromised individuals and pregnant women who show mortality rate up to 20% (CDC, 2016; Rothrock et al., 2019). *L. monocytogenes*, which is a facultative intracellular bacterium, is known to cause infections in both animals and humans. Food-borne infection controls are difficult because this agent is widely distributed in nature, can multiply in refrigerator environments, can tolerate a wide range of pH (4.3 to 9.6), and can multiply in high (10%) salt concentrations (Rocourt and Buchriser, 2007).

*L. monocytogenes* is a frequent contaminant of raw milk and an inhabitant of soil, water and other contaminated foods (Karthikeyan et al., 2015). It has been considered that nearly all cases of human listeriosis are foodborne and associated with consumption of contaminated dairy products, unwashed raw vegetables and under-cooked meat, seafood and poultry products (Dhama et al., 2015; Kurpas et al., 2018; Todd and Notermans, 2011). Recently, a noteworthy increase in chicken meat production is observed in Turkey like in other countries (TPMPBA, 2018). Consequently, the increase of infections and intoxications due to chicken meat may be due to improved rates of reporting and surveillance systems, or due to the increase in the population of elderly and immunocompromised people (EFSA, 2018). Birds may be an important vector and contributor to the contamination of the processing environment and transmission of *Listeria* to consumers via the food (Rothrock et al., 2017). According to the European Food Safety Authority (EFSA) report, 2.9% of all *L. monocytogenes* infections were caused by consumption of raw poultry products (EFSA, 2010).

The EFSA reported 2.480 confirmed invasive human cases of listeriosis in 2017. The EU notification rate was 0.48 cases per 100,000 population which was comparable with 2016. In the recent report published in 2018 by EFSA, *L. monocytogenes* was reported to be among the leading causes of food-borne infections with a mortality rate of 13.8% in 2017 (EFSA, 2018). Presence of *L. monocytogenes* in the food industry is a potential risk factor. Therefore, monitoring the microorganism through the food production and supply chain is of primary importance.

Determining the serotype distributions of *L. monocytogenes* isolates is important for understanding food-borne infections, for withdrawing the risk imposing products and for differentiation of pathogenic and non-pathogenic strains (Liu, 2013). *L. monocytogenes* was divided into 13 different serotypes according to its somatic and flagellar antigens, which are known as 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Strains of 1/2a, 1/2b, 1/2c and 4b are known to be important for listeriosis in humans (Doumith et al., 2004; Jamali et al., 2013; Nho et al., 2015). The most common (91.8%) serotypes causing listeriosis in humans are 1/2a and 4b (EFSA, 2015). Serotype 1/2a is frequently detected in various animal-sourced foods including chicken meat and products (Guerra et al., 2001; Orsi et al., 2011; Praakle-Amin et al., 2006).

The aim of the present study was to determine the prevalence and serotype distribution of *L. monocytogenes* in packaged chicken meats in Turkey.

## MATERIAL AND METHODS

### Food samples

Samples belonging to the five biggest poultry production companies were selected for investigation. These companies (A, B, C, D, and E) have slaughtering capacity of average 10.000 broilers/h and are marketing their products in most parts of the country. A total of 400 retail chicken meat samples (80 samples per company) were bought from different markets from April 2015 to January 2016. Each set of 80 samples consisted of 20 drumsticks, 20 breasts, 20 wings, and 20 whole chickens.

### Isolation and identification of *L. monocytogenes*

Each fresh sample was weighted in 25 g and the isolation of *L. monocytogenes* was conducted according to the classic culture method. This method includes two steps of pre-enrichment and selective enrichment in Half Fraser and Fraser Broth, respectively. After 225 ml of Half Fraser Broth (Merck 1.10398.0500) were added to samples (25 g), samples were homogenized in a stomacher (Interscience BagMixer 400 cc, France) for 2 to 3 min. The obtained homogenate was incubated for 24 h at 30°C for pre-enrichment. Following the pre-enrichment, 0.1 ml were taken from the culture and transferred into tubes containing 10 ml of Fraser Broth (Merck 1.10398.0500), then they were incubated for 24 h at 37°C. Inoculation into PALCAM Agar (Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin

Mannitol Agar, Oxoid CM0877-SR0150E) was conducted and agar plates were left for incubation at 37°C for 48 h. At the end of the incubation period, 1-5 *Listeria* suspected colonies (black-centered, gray green in color with a black halo) in PALCAM Agar were selected and were transferred into TSA-YE (Tryptone Soy Agar with Yeast Extract, Oxoid CM0131-YE). TSA-YE grown colonies were transferred to TSB (Tryptone Soy Broth, Oxoid CM0129) containing 20% glycerin and were stored in -20°C for advanced analyses (Jeyaletchumi et al., 2010).

**DNA extraction and PCR**

Five hundred µl from cultures of each *L. monocytogenes* isolate in TSB were transferred to microcentrifuge tubes and tubes were centrifuged for 5 min at 10000 rpm (VMR International Galaxy 16DH). Supernatants were discarded and 300 µl sterile distilled water was added. Afterwards, 300 µl K-Buffer (20 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.2% Sodium Dodecyl Sulphate (SDS)) and 5 µl Proteinase K (20 mg/ml) (Vivantis PC0712 (100 mg) were added to tubes. Tubes were incubated for 2 h at 56°C and were left in a water bath for 10 min at 95°C to inactivate proteinase K. Tubes were centrifuged for 10 min at 13000 rpm and the supernatants were discarded. A hundred µl sterile distilled water were added to remaining pellets and they were used as target DNAs in PCR.

Samples were analyzed first by using previously published primer sequences of *prs* and *prfA* genes, which are specific to *Listeria* spp. and *L. monocytogenes*, respectively. Afterwards, the serotypes of isolates were determined by multiplex PCR (mPCR) using specific primers *prs, lmo1118, lmo0737, orf2110* and *orf2819* (D'Agostino et al., 2004; Doumith et al., 2004).

For the detection of both *Listeria* spp. and *L. monocytogenes*, 50 µl PCR reaction mixtures were prepared. Each mixture was containing 5 µl 10X PCR Buffer (500 mM KCL, 100 mM Tris-HCl (pH 9.1) and 0.1% Triton X-100) (Vivantis, ViBufferA), 5 µl 25 mM $MgCl_2$ (Vivantis, 50 mM), 250 µM of each dNTP (Vivantis, NP2406, 100 mM), 20 pmol of each primer pair (Biomatik, Canada), 1.25 U Taq-polymerase enzyme (Vivantis PL1202, 500 U) and 5 µl (25 ng) of target DNA.

Serotyping of *L. monocytogenes* was conducted using the mPCR protocol of Doumith et al. (2004) in a thermal cycler device (Biorad-T100, Biorad, USA). The PCR protocol was as follows: Initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 sec, annealing at 53°C for 75 sec, extension at 72°C for 75 sec and a final extension at 72°C for 7 min. The protocol was conducted with 35 cycles. Reaction results were electrophoresed for 2 h in a 110 V in 1.5% agarose gel (Vivantis LE Grade Agarose Gel). Following the staining with ethidium bromide (10 mg/ml) (Merck 1.11608.0030), the gels were transilluminated (Vilber Lourmat Quantum ST4) to reveal DNA bands. *L. monocytogenes* ATCC 7644 (serotype 1/2c), RSKK 472 (serotype 1/2b), RSKK 471 (serotype 1/2a) and RSKK 475 (serotype 4b) were used as positive controls, while distilled water was used as negative control.

**Statistical analysis**

The statistical analyses were performed SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was used for analyzing the data obtained from the groups (products and companies) about the contamination with *L. monocytogenes.* The differences were considered significant at $p<0.05$.

**RESULTS**

As shown in Table 1, 267 (66.75%) of the investigated chicken meat samples were detected by using classical culture method as suspect for *Listeria* spp. It is known that detection of non-pathogenic *Listeria* spp. can be challenging using classical culture methods due to the frequent presence of non-*Listeria* spp. background flora colonies that are β-glucosidase-positive (Angelidis et al., 2015). Hence, the PCR results for these isolates verified that a smaller fraction (62.75%) of the samples (251 in 400) were actually contaminated with *Listeria* spp.

Of the 251 suspect strains, 106 strains were determined in PCR as *L. monocytogenes.* The prevalence of *L. monocytogenes* in the investigated drumstick, breast, wing and whole chicken products were 47%, 15%, 35% and 9%, respectively (Table 1).

The highest isolation frequency for *L. monocytogenes* was noted in samples from company B (52.5%), whereas the lowest in company A (2.5%). Differences between company B and others (A, C, D, and E) were found as statistically significant ($p<0.05$). The *L. monocytogenes* isolation frequency in samples from companies C, D, and E were 32.5%, 18.8% and 26.3%, respectively. Differences between these three companies were found to be non-significant ($p>0.05$) (Table 2).

**Table 1.** Distributions of *Listeria* spp., *L. monocytogenes* and serotypes in chicken meats

| Product | No. of samples | Culture results* | *Listeria* spp. | *L.monocytogenes* | *L. monocytogenes* serotype distribution (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 1/2a | 1/2b | 1/2c | 4b |
| Drumstick | 100 | %73 (73/100) | % 71 (71/100) | % 47 (47/100) | %93.6 (44/47) | %2.1 (1/47) | %2.1 (1/47) | %2.1 (1/47) |
| Breast | 100 | %67 (67/100) | % 66 (66/100) | % 15 (15/100) | %100 (15/15) | 0 | 0 | 0 |
| Wing | 100 | %82 (82/100) | % 80 (80/100) | % 35 (35/100) | %91.4 (32/35) | 0 | %5.7 (2/35) | %3.1 (1/35) |
| Whole Chicken | 100 | %45 (45/100) | % 34 (34/100) | % 9 (9/100) | %100 (9/9) | 0 | 0 | 0 |
| Total | 400 | %66.75 (267/400) | %62.75 (251/400) | %26.5 (106/400) | %94.3 (100/106) | %0.9 (1/106) | %2.8 (3/106) | %1.9 (2/106) |

* Presumptive-positive results

**Table 2.** *L. monocytogenes* distribution according to company and product type

| Product | Companies | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | Total |
| **Drumstick** | %0$^{d}$ (0/20) | %100$^{Aa}$ (20/20) | %70$^{Ab}$ (14/20) | %20$^{d}$ (4/20) | %45$^{Ac}$ (9/20) | 47/100 |
| **Breast** | %5 (1/20) | %10$^{C}$ (2/20) | %15$^{B}$ (3/20) | %10 (2/20) | %35$^{A}$ (7/20) | 15/100 |
| **Wing** | %5$^{b}$ (1/20) | %85$^{Ba}$ (17/20) | %35$^{Bb}$ (7/20) | %30$^{b}$ (6/20) | %20$^{Ab}$ (4/20) | 35/100 |
| **Whole chicken** | %0 (0/20) | %15$^{C}$ (3/20) | %10$^{B}$ (2/20) | %15 (3/20) | %5$^{B}$ (1/20) | 9/100 |
| **Total** | **%2.5 2/80** | **%52.5 42/80** | **%32.5 26/80** | **%18.8 15/80** | **%26.3 21/80** | **%26.5 106/400** |

$^{A-C}$ Differences between mean values of different letters are significant in the same column
$^{a-d}$ Differences between mean values of different letters are significant in the same row

Four important serotypes of *L. monocytogenes* for human listeriosis (1/2a, 1/2b, 1/2c, and 4b) were investigated in *L. monocytogenes* isolates (n=106). Serotype 1/2a was determined as predominant with 94.3% in the isolates from chicken meats. Other determined serotypes in the isolates were 1/2c (n=3; 2.8%) and 1/2b (n=1; 0.9%). As for their distribution, *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c and 4b were determined in drumstick samples, and serotypes 1/2a, 1/2c and 4b were determined in wing samples (Table 1). All serotypes determined in breast and whole chicken samples were 1/2a. Serotype 1/2a, which was determined as predominant, was detected in 93.6% of drumsticks, 100% of breasts, 91.4% of wings and in 100% of whole chickens.

## DISCUSSION

*L. monocytogenes* is an important pathogenic microorganism in terms of public health. Acquisition of listeriosis is mainly due to consumption of contaminated (mostly ready-to-eat) food (Churchill et al., 2019; Lomonaco et al., 2015).

According to the other studies, the *L. monocytogenes* presence in different chicken meat products (drumstick, breast, and wings) in the world was reported to be between 7.14% in Malaysia and 71% in Spain (Goh et al., 2012; López et al., 2013). The prevalence of this pathogen in chicken meats was reported as varying between 8.4% and 38.4% in Turkey (Cetinkaya et al., 2015; Guven and Patir, 1998).

Chicken meat contamination with *L. monocytogenes* is known to be significantly increased during both slaughtering and processing of viscera and parts. Product comparisons in the study revealed that the *L. monocytogenes* isolation frequency in drumsticks was higher than other tested products. Drumsticks

are most preferred parts in our country and are used commonly in oven-baked meals. Companies lead products to more than one processing stage to make their products more appealing to the consumer and to improve long-term customer loyalty. These approaches may increase the level of cross contamination. The *L. monocytogenes* prevalence in drumsticks (47%) in this study was found higher than previously reported estimates of 11.27% (Goh et al., 2012) and 33.3% (Erol et al., 1999). This difference can be attributed to the company conditions, processing capacity, cross-contamination risks during the slaughtering and to the holding time of food before marketing (Goh et al., 2012).

In this study, the prevalence of *L. monocytogenes* in packaged chicken breast samples was 15% (Table 1). This was found to be similar to the previously reported estimates of 16.6% (Erol et al., 1999) and 18% (Soultos et al., 2003). Our estimate, however, was found lower than that reported (42.03%) in breast samples from wet markets by Goh et al. (2012). This difference is thought to be because of packaged and without skin breasts provided in markets.

The *L. monocytogenes* isolation frequency in wing samples was higher than those previously reported, i.e., 20% (Erol et al., 1999) and 18% (Soultos et al., 2003). Elmali et al., (2015) reported summer, autumn, winter, and spring *L. monocytogenes* prevalences in packaged wing samples as 60%, 73.3%, 33.3% and 13.3% respectively. In the study, *L. monocytogenes* in packaged wings was determined as 35%. Even though seasonal frequency was not investigated, most of the samples in the study were taken in the winter season. This is in agreement with the winter season findings of Elmali et al. (2015). In a study by Ayaz and Erol (2009) where *L. monocytogenes* presence was investigated in turkey meats, it was reported that seasonal effect was not present due to the psychrotrophic character of the bacteria.`

In addition to reports from EFSA and CDC, various studies reported that most of the human listeriosis cases (more than 95%) are due to serotypes 1/2a, 1/2b, 1/2c, and 4b (CDC, 2014; Doumith et al. 2004; EFSA, 2015; Jamali et al. 2013; Nho et al. 2015). According to the report published by EFSA, the most commonly responsible serotype of human listeriosis cases is 1/2a (57.5%), which is followed by 4b, 1/2b, 1/2c, 3a and 3b (EFSA, 2015). In our study, the predominant serotype of *L. monocytogenes* in chicken meats (drumsticks, breasts, wings and whole chickens) was 1/2a (94.3%), which was followed by 1/2c, 4b and 1/2b (Table 1). Previous studies similarly reported that 1/2a is the most common serotype (Cetinkaya et al., 2014; Erol et al., 1999; Guerra et al., 2001; Praakle-Amin et al., 2006; Siriken et al., 2014).

Nevertheless, there are other studies with different findings. In a study conducted in the US, it was reported that 1/2b is the most common serotype, which is closely followed by serotype 4b (Zhang et al., 2007). In another study conducted in Iran, it was reported that serotype 4b is the most common, which is closely followed by serotype 1/2a (Fallah et al., 2012). In Turkey, infections by *L. monocytogenes* were reported as sporadic cases (Vardar et al., 2011); however, insufficient data is available about serotypes in human listeriosis cases.

## CONCLUSIONS

In conclusion, the prevalence of *L. monocytogenes* in fresh chicken meats as a potential risk factor for humans was highlighted in this study. Since 26.5% of the investigated samples in the study were contaminated with *L. monocytogenes* and the most significant serotype in food-borne listeriosis cases, serotype 1/2a, was detected, it can be concluded that chicken meats may pose risk for public health. The present study provides an insight on the prevalence and serotype distribution of *L. monocytogenes* in chicken meats in Turkey. A baseline information is represented here, for further studies which may aim to improve microbiological safety procedures for raw foods including *L. monocytogenes*. To prevent listeriosis cases due to chicken meats, it is recommended to take preventive measures in general hygiene and disinfection applications through the production line and to take care in properly cooking such products.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

# Epidemiological evaluation of subclinical mastitis of dairy cows in Greece

**K. Themistokleous, I. Karagiannis, C. Boscos, N. Panousis, E. Kiossis**

*Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 54627, Thessaloniki, Greece*

**ABSTRACT.** Subclinical mastitis, diagnosed by elevated somatic cell count (SCC) in milk, is an important monitoring parameter of dairy cows' udder health, related to their productivity and welfare. The present retrospective study aims to evaluate the epidemiology of subclinical mastitis (SCM) among the 37 herds of the Holstein Association of Greece participating in the milk quality recording system "IΩ", from the start of 2015 until the end of 2018. The herds' inclusion criterion was the consistency of monthly SCC recording throughout at least one full year between 2015 and 2018, with a maximum interval of 61 days between two consecutive monthly SCC recordings. Twenty-six herds (8630 cows) in 2015, thirty herds (10763 cows) in 2016, thirty herds (10945 cows) in 2017 and twenty-six herds (9597 cows) in 2018 were included. The prevalence of SCM and chronic SCM, the incidence rate of new cases of SCM, as well as the average somatic cell score and bulk tank milk SCC were determined for each of the four years. The results indicate a progressive deterioration of udder health from the onset of the cow's productive life until culling. A year-over-year increase in the number of cows with subclinical mastitis led to an overall SCM prevalence of 34.5%, chronic SCM prevalence of 26.9% and a bulk tank milk SCC of 463000 cells/mL, in 2018. The average somatic cell score, a base 2 logarithm of individual cow's SCC, was found persistently above the subclinical mastitis indicative cut-off in all four years, with a peak in 2018. At herd level, the incidence rate of new SCM cases was 12 new cases / 100 cows / month; the highest incidence rate was observed in the early lactation stage group (1-60 days-in-milk), in all four years, reaching a peak of 31 new cases / 100 cows / month, in 2018. In 2018, prevalence of heifers' SCM and chronic SCM was 23.4% and 16.9%, respectively. Despite the adequate average 305-days milk yield (9608 kg in 2018), the results were indicative of poor udder health status, pointed out by reduced duration of cows' productive life (less than 3 lactations) and lower milk quality (elevated SCC). The severity and wide spreading of subclinical mastitis in Greek dairy herds highlights the necessity of a national mastitis control program, aiming to improve the productive efficacy, management decisions accuracy and quality of produced milk.

***Keywords:*** subclinical mastitis; somatic cell count; dairy cows; epidemiology; udder health

*Corresponding Author:*
Themistokleous K., Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 11 St. Voutyra str., 546 27 Thessaloniki, Greece
E-mail address: konsthem@vet.auth.gr

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## INTRODUCTION

Udder health status of dairy cows is related to their productivity and welfare. Thus, it is crucial both for the consumer and for the dairyman, regarding the production of high-quality milk, duration of productive life, reduction of antibiotic residues, as well as the profitability of the livestock business. A study of the National Animal Health Monitoring System (NAHMS) in USA revealed that udder health problems is the number one reason for culling a dairy cow (26.9% ±0.5%) (USDA / NAHMS, 2002). The importance of this subject has led to a worldwide increase of the attention paid to the establishment and development of udder health monitoring programs (Schukken et al., 2003). Subclinical mastitis, new and chronic, is an important monitoring parameter of udder health (Schukken et al., 2008), as this disease leads to the reduction of milk yield (Archer et al., 2013), higher risk of clinical mastitis (Rupp et al., 2000), higher chances of premature culling (De Vliegher et al., 2005) and, consecutively, economic losses (Hamann, 2005).

Subclinical mastitis (SCM) is the presence of an infection without apparent clinical changes (condition of the udder or milk secretion) (Blowey and Edmondson, 2010). The diagnosis of SCM is based on the recognition of the mammary inflammatory response against the infection (Shook et al., 2017). A widely used tool for this purpose is somatic cell count (SCC) (Dohoo and Leslie, 1991; Schukken et al., 2003; Ruegg and Pantoja, 2013). Somatic cells take part in the udder defence mechanisms (Pillai et al., 2001) and their presence reflects the inflammatory response to an intra-mammary infection (IMI) or some other trigger of the immune system (Schukken et al., 2003). Longitudinal data of bulk tank milk SCC (BTSCC) over time can be an indicator of the udder health status at herd level. BTSCC is highly correlated with 305-days milk yield and it can be associated with the prevalence of cows that produce milk with elevated SCC (Smith, 1996; Barkema et al., 1998). A more appropriate parameter to summarize herd's average SCM situation is the arithmetic average test-day SCC from individual cow milk samples, as well as a base 2 logarithmic conversion of SCC, the somatic cell score (SCS), which is providing statistical superiority (Shook, 1993; Lievaart et al., 2007).

Various thresholds have been suggested for the classification of infected and non-infected cows. It is repeatedly shown that a cut-off of approximately 200000 to 250000 cells per mL is optimal to reduce diagnostic error (Dohoo and Leslie, 1991; Schepers et al., 1997). Many studies used a cut-off of 200000 cells/mL (Schukken et al., 2003; De Vliegher et al., 2004; Svensson et al., 2006; Fouz et al., 2010; Madouasse et al., 2012; Lam et al., 2013; Fauteux et al., 2014; Santman-Berends et al., 2016), while especially for heifers, IMI cut-offs of 150000 cells/mL (Santman-Berends et al., 2016) and 100000 cells/mL (Bludau et al., 2014) have been used. In our study, due to the available data, a preset threshold of 250000 cells/mL was utilized. These cut-offs are a practical threshold under field conditions and not the ultimate goal for udder health and best quality milk production (Schukken et al., 2003).

This four-year retrospective study aims to introduce an epidemiological evaluation of SCM among Holstein dairy cows in Greece, from 2015 until 2018. Utilizing the available data from "IΩ" recording system, provided by the Holstein Association of Greece (HAOG), our study presents: subclinical mastitis prevalence, chronic subclinical mastitis prevalence, subclinical mastitis incidence rate, as well as somatic cell score (SCS), bulk tank milk somatic cell count (BTSCC) and some overall observations regarding the productive efficacy of the population of dairy cows belonging to HAOG, during the study period.

## MATERIALS AND METHODS

### Study population

Among the 84 herd-members of HAOG, 37 took part in the monthly milk quality recording system, called "IΩ". From those 37 herds, the ones included in the current study met the following criterion: they were consistently recording monthly SCC for at least one full year between 2015 and 2018, having a maximum interval of 61 days between two consecutive monthly SCC recordings. Based on the above criterion, 26 herds (8630 cows) were included in the study in 2015, 30 herds (10763 cows) in 2016, 30 herds (10945 cows) in 2017 and 26 herds (9597 cows) in 2018. In total, 39424 test-month recordings and 1568 annual recordings were utilized from "IΩ" during this four-year study.

### Available data

On a monthly basis, milk SCC on test-day was calculated from individual cow milk samples, as well as bulk tank milk samples collected from each of the aforementioned herds. The following data were obtained from "IΩ":

- Monthly, the number of milked cows per herd, per number [1st ("heifers"), 2nd and 3rd lactation] and per stage of lactation [1-60 ("early lactation"), 61-120, 121-180, ≥181 days-in-milk], provided as a preset grouping by "IΩ".

- Monthly, the total number of infected (SCC above the cut-off on test-day) and new cases of infected cows per herd, per number and per stage of lactation.

- Monthly, the average individual SCC per herd, per number and per stage of lactation, as well as mean bulk tank milk SCC (BTSCC) of the herd on test-day.

- Annually, the total number of milked cows per herd, the herd average age at first calving (months), the calving interval (days) and the number of lifetime lactations per cow. Also, the average individual 305-days milk yield (kg) and duration of lactation period (days) per herd, per number and per stage of lactation.

**Definitions and epidemiological analysis**

Diagnosis of subclinical mastitis

Subclinical mastitis diagnosis was based on individual cow milk SCC results at monthly test-day. "Infection" was defined as SCC value above the threshold for normal milk SCC concentration (Schukken et al. 2008), on test-day. The threshold was preset by "IΩ" at 250000 cells/mL.

Somatic cell score

A base 2 logarithmic transformation of the SCC calculated as $\log_2(SCC/100) +3$.

Average subclinical mastitis prevalence

Subclinical mastitis prevalence was calculated as the monthly average percentage of infected cows on test-day, per herd, per number and per stage of lactation, for each of the years 2015-2018. This monthly percentage is the ratio of infected (SCC above the cut-off) lactating cows to the total number of lactating cows participating in the test-day recording of a certain month. (Santman-Berends et al., 2016).

Average chronic subclinical mastitis prevalence

The number of chronically infected cows of a certain month was calculated by the subtraction of the new cases of infected cows from the total number of infected cows on the monthly test-day, both provided by "IΩ" system. Herd average chronic SCM prevalence (%) was calculated as the average monthly percentage of chronically infected cows on test day, per herd, per number and per stage of lactation, for each of the years 2015-2018. This monthly percentage is the ratio of chronically infected lactating cows to the total number of lactating cows participating in the test-day milk recording of a certain month (Santman-Berends et al., 2016).

Subclinical mastitis incidence rate

Subclinical mastitis incidence rate was calculated per 100 cows at risk / month, and was the number of new SCM cases divided by days at risk (DAR), multiplied by 30 days and 100 cows, as follows:

(new infections / DAR) x 30 days x 100 cows

DAR refers to the days of a certain month, during which the lactating cow was exposed to an intra-mammary infection risk. Cows that maintained a SCC below the threshold all month long, despite the exposure to the risk, had 30 DAR. Given the fact that the only available data was monthly (and not daily) recordings, the approximate method was used (Dohoo et al., 2003), assuming that a new infection happened in the middle of each month, i.e. 15 DAR for newly infected cows.

Annual data

Provided by "IΩ" system, herd annual data were utilized for the calculation of the annual number of milked cows participating in the study during each year from 2015 until 2018, as well as the annual average age at 1st calving (months), calving interval (days), number of lifetime lactations / cow and annual herd average BTSCC (cells/mL). The annual average 305-days milk yield / cow, SCS and duration of lactation period (days) were calculated per herd and per number of lactations. Furthermore, annual average SCS was calculated per stage of lactation, from 2015 to 2018.

The aforementioned udder health evaluation parameters were processed and presented via descriptive statistics, using Stata 13.1® (StataCorp LLC, College Station, Texas, 2014) and Microsoft Excel® (Microsoft Office 365, Microsoft©).

## RESULTS

**Annual data**

The average annual herd data evaluated in this

four-year study are presented in Table 1. Average age at first calving ranged between 27.5 (±2.2) and 28.2 (±2.4) months and remained above the 27 months (upper optimal threshold) during all four years, progressively decreasing from 2015 to 2018. A minor decrease of 9 days was observed for herd average calving interval, with a nadir of 447 (±34) days in 2018. Herd average BTSCC in 2015 was 385000 cells/mL, although there was a considerable year over year increase up to 463000 cells/mL in 2018. Average number of lifetime lactation periods / cow was increased by 0.21 compared to 2015, peaking in 2017 (2.91 ±0.35) and remaining stable in 2018 (2.91 ±0.32), but still lower than the minimum target of 3 lifetime lactations. Herd average 305-days milk yield / cow, presented in Table 2, increased year over year with a peak of 9608 (±1609) kg in 2018, 645 kg higher than 2015. Interestingly, during the four-year period, in 46% of the herds the average 305-days milk yield / cow of the 3rd lactation was lower than that of the 2nd lactation. Finally, herd average duration of lactation was decreased by 14 days from 2015 to 2016, then increased by 10 days from 2016 to 2017 and remained unaltered in 2018 at 347 (±35) days (Table 2).

**Table 1.** Annual herd data of milked cows, average age at first calving, average number of lifetime lactations, average calving intervals and average bulk tank milk somatic cell count (BTSCC), during 2015-2018 in Greece.

| year (number of herds) | number of milked cows | average age at 1st calving (months) | average number of lifetime lactations | average calving interval (days) | average BTSCC (x1000 cells/mL) |
|---|---|---|---|---|---|
| 2015 (n=26) | 8630 | 28.2 (± 2.4) | 2.70 (± 0.28) | 456 (± 24) | 385 (± 142) |
| 2016 (n=30) | 10763 | 28.1 (± 2.3) | 2.74 (± 0.32) | 449 (± 28) | 396 (± 180) |
| 2017 (n=30) | 10945 | 27.6 (± 2.3) | 2.91 (± 0.35) | 448 (± 29) | 416 (± 178) |
| 2018 (n=26) | 9597 | 27.5 (± 2.2) | 2.91 (± 0.32) | 447 (± 34) | 463 (± 165) |

(±): standard deviation

**Table 2.** Average somatic cell score (SCS), 305-days milk yield (kg) and duration of lactation period (days), per herd & per lactation period (1st, 2nd, 3rd LP) groups, during 2015-2018 in Greece

| year (number of herds) | | SCS | average 305-days milk yield (kg) | average duration of lactation period (days) |
|---|---|---|---|---|
| 2015 | Herd | 4.8 (±0.5) | 8963 (±1308) | 351 (±25) |
| (n=26) | 1st LP | 4.2 (±0.5) | 8461 (±1269) | 365 (±36) |
| | 2nd LP | 4.7 (±0.7) | 9284 (±1361) | 359 (±27) |
| | 3rd LP | 5.6 (±0.6) | 9527 (±1279) | 343 (±41) |
| 2016 | Herd | 4.8 (±0.6) | 9131 (±1346) | 337 (±24) |
| (n=30) | 1st LP | 4.4 (±0.8) | 8573 (±1217) | 358 (±41) |
| | 2nd LP | 4.6 (±0.8) | 9429 (±1489) | 337 (±36) |
| | 3rd LP | 5.5 (±0.6) | 9350 (±1795) | 338 (±38) |
| 2017 | Herd | 4.9 (±0.7) | 9429 (±1404) | 347 (±27) |
| (n=30) | 1st LP | 4.4 (±0.9) | 8807 (±1245) | 377 (±69) |
| | 2nd LP | 4.8 (±0.8) | 9856 (±1626) | 354 (±27) |
| | 3rd LP | 5.6 (±0.5) | 9935 (±1491) | 351 (±59) |
| 2018 | Herd | 5.1 (±0.6) | 9608 (±1609) | 347 (±35) |
| (n=26) | 1st LP | 4.5 (±0.6) | 9190 (±1386) | 366 (±47) |
| | 2nd LP | 5.0 (±0.8) | 9849 (±1941) | 359 (±44) |
| | 3rd LP | 5.8 (±0.5) | 9973 (±1538) | 344 (±45) |

(±): standard deviation

**Somatic cell score**

Herd average SCS, presented in Table 2, was steadily above the 4.0 SCM-indicative threshold during all four years, both at herd level and at number and stage of lactation levels. Average SCS appeared elevated even from the first lactation (4.2 in 2015, 4.4 in 2016, 4.4 in 2017 and 4.5 in 2018), increased in the second and peaked in the third lactation (5.8 in 2018). Early lactation SCS, presented in Table 3, ranged from 4.7 (2015, 2016) to 5.1 (2018), exceeding the aforementioned threshold during all four years of the study. Average SCS remained above the threshold throughout the whole lactation and it reached its highest score in the last stage of lactation (≥181 days-in-milk).

**Subclinical mastitis and chronic subclinical mastitis prevalence**

Average subclinical mastitis prevalence was consistent with the SCS results at herd and at number and stage of lactation levels, as seen in Table 4. In the four-year period, the average herd SCM prevalence was between 29.6% and 34.5%, increasing from 2016 until 2018. Herd average chronic SCM prevalence was between 22.1% and 26.9% during the whole study period, showing an increase during the last 3 years. Interestingly, both SCM prevalence and chronic SCM prevalence were noticeably high from 1st lactation, reaching 23.4% and 16.9%, respectively, in 2018. This situation deteriorated as lactation number increased, reaching its' peak within the 3rd lactation (average SCM prevalence 46% and average chronic SCM prevalence 37% in 2018). Average SCM prevalence in all four years was elevated even from early lactation (29.6% in 2018) and reached its highest level in the last stage of lactation (39.2% in 2018). Finally, average chronic SCM prevalence during 2015-2018 ranged between 5.3 and 6.6 in early lactation and increased with the progress of lactation, reaching a peak at the last stage of lactation (35.6% in 2018).

**Subclinical mastitis incidence rate**

The highest SCM incidence rate was observed in early lactation, also increasing from 27 to 31 new cases / 100 lactating cows / month in the last three years. In the later lactation stages, this rate was reduced by almost three times. Regarding the number of lactations, the SCM incidence rate gradually increased from 1st to 3rd one and peaked in the 3rd lactation. Overall herd average SCM incidence rate in 2018 was 12 new cases / 100 lactating cows / month (Table 4).

**Table 3.** Average somatic cell score (SCS) per lactation stage groups (≤60, 61-120, 121-180, ≥181 days-in-milk, DIM) during 2015-2018 in Greece

| | Lactation stage (DIM) | SCS |
|---|---|---|
| 2015 | ≤60 | 4.7 (±0.6) |
| (n=26) | *61-120* | 4.6 (±0.7) |
| | *121-180* | 4.8 (±0.6) |
| | ≥181 | 5.2 (±0.5) |
| 2016 | ≤60 | 4.7 (±0.6) |
| (n=30) | *61-120* | 4.6 (±0.8) |
| | *121-180* | 4.7 (±0.6) |
| | ≥181 | 5.1 (±0.6) |
| 2017 | ≤60 | 4.8 (±0.6) |
| (n=30) | *61-120* | 4.8 (±0.8) |
| | *121-180* | 4.8 (±0.8) |
| | ≥181 | 5.2 (±0.7) |
| 2018 | ≤60 | 5.1 (±0.6) |
| (n=26) | *61-120* | 5.0 (±0.7) |
| | *121-180* | 5.2 (±0.6) |
| | ≥181 | 5.3 (±0.6) |

(±): standard deviation

**Table 4.** Subclinical mastitis prevalence (SCMP - %), chronic subclinical mastitis prevalence (chronic SCMP - %) and subclinical mastitis incidence rate (SCMIR - number of new cases per 100 lactating cows per month), per herd, number of lactation period (1st, 2nd, 3rd LP) and lactation stage (≤60, 61-120, 121-180, ≥181 days-in-milk, DIM) groups, during 2015-2018 in Greece

| | 2015 (n=26) | | | | | | 2016 (n=30) | | | | | | 2017 (n=30) | | | | | | 2018 (n=26) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SCMP | | chronic SCMP | | SCMIR | | SCMP | | chronic SCMP | | SCMIR | | SCMP | | chronic SCMP | | SCMIR | | SCMP | | chronic SCMP | | SCMIR | |
| | % | SD | % | SD | # | SD | % | SD | % | SD | # | SD | % | SD | % | SD | # | SD | % | SD | % | SD | # | SD |
| Herd | 30.2 | ±7.9 | 23.7 | ±7.5 | 10 | ±2 | 29.6 | ±9.2 | 22.1 | ±8.2 | 11 | ±4 | 32.4 | ±12,6 | 25.1 | ±11.6 | 11 | ±4 | 34.5 | ±11.6 | 26.9 | ±11.1 | 12 | ±4 |
| LP: | | | | | | | | | | | | | | | | | | | | | | | | |
| 1st | 19.4 | ±5.4 | 14.3 | ±5.2 | 6 | ±2 | 20.8 | ±9.5 | 14.6 | ±9.1 | 8 | ±3 | 23.3 | ±13,4 | 17.0 | ±11.7 | 9 | ±6 | 23.4 | ±10.3 | 16.9 | ±9.6 | 9 | ±3 |
| 2nd | 27.7 | ±10.0 | 21.0 | ±9.2 | 10 | ±3 | 26.5 | ±12.0 | 19.3 | ±10.9 | 10 | ±4 | 28.9 | ±12,6 | 22.1 | ±12.0 | 11 | ±7 | 32.6 | ±16.5 | 25.3 | ±15.5 | 12 | ±7 |
| 3rd | 41.8 | ±10.2 | 33.7 | ±10.1 | 14 | ±4 | 40.5 | ±12.2 | 31.4 | ±11.7 | 15 | ±6 | 42.0 | ±14,2 | 33.6 | ±13.5 | 15 | ±6 | 46.0 | ±12.7 | 37.0 | ±12.3 | 17 | ±7 |
| DIM: | | | | | | | | | | | | | | | | | | | | | | | | |
| ≤60 | 26.7 | ±6.9 | 6.1 | ±3.1 | 27 | ±11 | 26.7 | ±7.8 | 5.6 | ±3.7 | 27 | ±9 | 27.9 | ±11,7 | 5.3 | ±2.7 | 29 | ±11 | 29.6 | ±9.3 | 6.6 | ±3.7 | 31 | ±12 |
| 61-120 | 25.6 | ±10.5 | 18.1 | ±9.0 | 10 | ±6 | 23.5 | ±8.9 | 16.5 | ±7.9 | 9 | ±4 | 26.7 | ±11,4 | 19.0 | ±10.2 | 10 | ±5 | 28.4 | ±10.9 | 21.1 | ±9.7 | 10 | ±5 |
| 121-180 | 26.9 | ±9.4 | 22.3 | ±9.9 | 7 | ±4 | 26.9 | ±9.8 | 21.7 | ±9.5 | 8 | ±4 | 29.1 | ±13,7 | 24.4 | ±13.0 | 8 | ±5 | 31.9 | ±10.8 | 26.0 | ±10.4 | 9 | ±4 |
| ≥181 | 34.6 | ±9.0 | 31.5 | ±9.5 | 5 | ±2 | 33.3 | ±9.9 | 29.3 | ±9.7 | 6 | ±3 | 37.1 | ±14,7 | 33.3 | ±14.9 | 6 | ±2 | 39.2 | ±13.8 | 35.6 | ±14.2 | 6 | ±3 |

SD (±): Standard Deviation

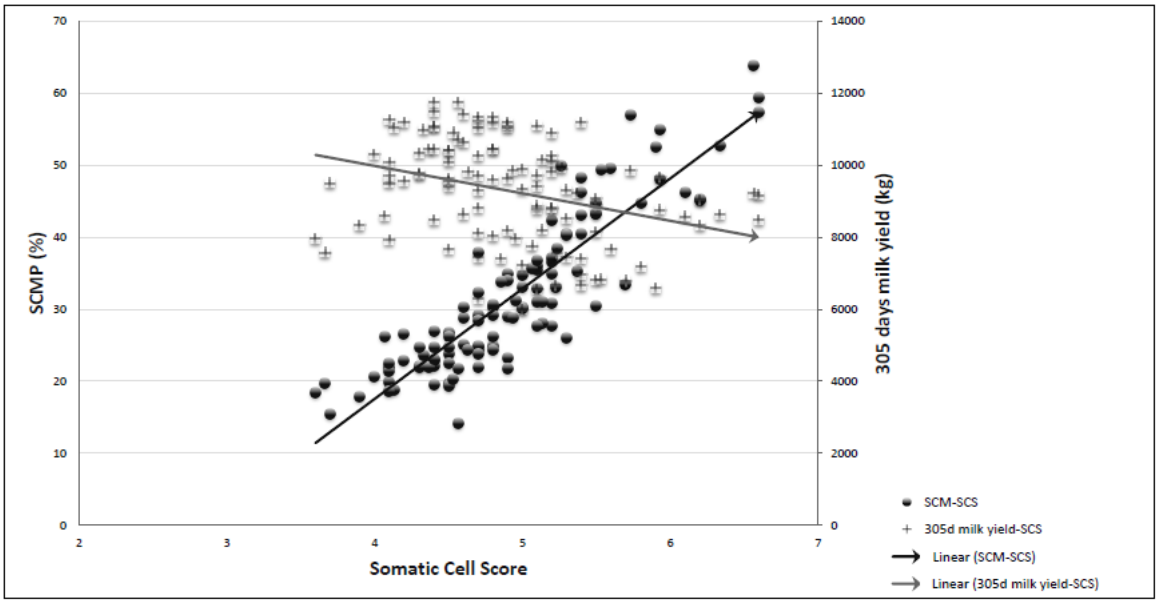
### Subclinical mastitis: year over year epidemiology

The epidemiology of subclinical mastitis presented a year over year deterioration. Therefore, the results of 2018 appeared worse than 2015. More precisely, herd average BTSCC and herd average SCS were higher by 78000 cells/mL (Table 1) and by 0.3 points (Table 2), respectively, in 2018 compared to 2015. Herd average SCM prevalence in 2018 was 4.9% higher than 2016 and herd average chronic SCM prevalence was 4.8 % higher than 2016. Finally, according to herd average SCM incidence rate in 2018, 12 new cases per 100 lactating cows (2 more new cases than 2015) were added to the existing chronic cases each month (Table 4).

## DISCUSSION

The aim of this study was to evaluate the epidemiology of subclinical mastitis among dairy herds of the Holstein Association of Greece, during the period 2015-2018. The necessary inclusion criterion of consistent data recording could be a selection bias, firstly because precise and meticulous dairymen are related to better herd performance and, secondly, because low milk SCC can be associated with farmer's management style (Barkema et al., 1999; Barnouin et al., 2004). The subclinical mastitis epidemiology presented in the results of this study could be summarized as high overall subclinical mastitis prevalence, high chronic subclinical mastitis prevalence and high subclinical mastitis incidence rate, as well as consequently elevated SCS and BTSCC.

**Figure 1.** Scatter diagram with linear trend lines of the relationship between somatic cell score (SCS), subclinical mastitis prevalence (SCMP - %) and 305 days milk yield (305d milk yield – kg), during 2015-2018 in Greece



Regarding the relationship between herd average SCM prevalence and herd average SCS (Figure 1), including all herds that took part in the study during the four-year period, the increasing linear trend line of "SCM prevalence – SCS" confirmed the expectedly positive relationship between SCC and intramammary infection. Elevated average SCS of individual cows at herd level, as well as at number and stage of lactation groups (Tables 2, 3) is closely related with the elevated SCM prevalence, as each 1-point increase in SCS can be associated with a 9.1% increase in the prevalence of intra-mammary infection (Shook et al., 2017).

The decreasing linear trend line of "305-days milk yield – SCS" (Figure 1) shows that high somatic cell score was negatively related to milk production. It is known that cows with high somatic cell count (and, consequently, SCS) produce a lower milk volume than cows with low SCC, and that there is a negative correlation between total milk volume produced and the somatic cell count per milliliter of milk produced. Intramammary infections (leading to high SCC) may reduce milk yield through chronic damage to mammary secretory cells, but even in short-duration infections with no permanent damage, metabolic resources may be diverted from milk production to immune defense (Green et al., 2006).

Interestingly, 46% of the herds during the four-year period of our study had a lower 305-days milk yield / cow in the 3rd than in the 2nd lactation period. This result is contrary to the normal milk production pattern, according to which milk production is increasing from 1st to the 3rd lactation (Clark, 1924; Michel, 1994). This abnormality can be attributed to the deterioration of udder health from 1st to 3rd lactation groups, as observed by the elevated subclinical mastitis prevalence, especially the chronic one, and somatic cell score above the SCM-indicative threshold throughout all four years of the study (Tables 2, 4). Other factors that can lead to decreased milk production as the number of lactations increases are: high prevalence of lameness, poor reproduction, nutritional and managerial errors. However, due to the retrospective nature of the study, there were no data available for those factors and, therefore, their co-effect cannot be evaluated. Table 5. National levels of subclinical mastitis prevalence (%), heifers subclinical mastitis prevalence (%) and 305-days milk yield (kg), compared to the results of 2018 in Greece.

**Table 5.** National levels of subclinical mastitis prevalence (%), heifers subclinical mastitis prevalence (%) and 305-days milk yield (kg), compared to the results of 2018 in Greece

| **Subclinical Mastitis Prevalence** | | | | **Heifers Subclinical Mastitis Prevalence** | | | | **305-days Milk Yield ††** | |
|---|---|---|---|---|---|---|---|---|---|
| Author | Country | SCC cut-off | SCMP | Author | Country | SCC cut-off | Heifers SCMP | Country | 305d milk yield (kg) |
| *Skrzypek et al., 2004* | Poland | 400000 | 21.3% | *Fox et al., 1995* | USA | 200000 | 36% | Germany | 9219 |
| *Madouasse et al., 2010* | England & Wales | 200000 | 25% | *De Vliegher et al., 2004b* | Belgium | 200000 | 27.5% † | France | 9042 |
| *Lam et al,. 2013* | Netherlands | 200000 | 22% | *Svensson et al., 2006* | Sweden | 200000 | 18.1% ύ | Sweden | 10325 |
| *Fauteux et al,. 2014* | Canada | 200000 | 26% | *Parker et al., 2007* | New Zealand | 200000 | 13.3% | Italy | 9980 |
| *Shook et al., 2017* | USA | - | 37.6% * | *Fouz et al., 2010* | Spain | 200000 | 21.7% § | Spain | 10152 |
| | | | | *Santman-Berends et al., 2012* | Netherlands | 150000 | 25.5% Π | Netherlands | 9958 |
| | | | | *Bludau et al., 2014* | Switzerland | 100000 | 20.6% | Switzerland | 8813 |
| Themistokleous et al., 2019 | **Greece** | 250000 | 34.5% | Themistokleous et al., 2019 | **Greece** | 250000 | 23.4% | **Greece** | 9608 |

*: % of intramammary infection (pathogen specific tests), †: 5-14 days in milk (DIM), ύ: first milking, §: 5-37 DIM, Π: first 100 DIM, ††: Data obtained from the European Holstein & Red Holstein Confederation, except Greece (Themistokleous et al., 2019).

Despite the satisfactory, in comparison with other European countries (E.H.R.H.C. 2017), average 305-days milk yield / cow (Table 5), the results discussed below indicate a progressive deterioration of udder health from the onset of productive life until culling. It was observed here that the average BTSCC in 2018 was 463000 cells/mL, approximately 78000 cells/mL higher than in 2015. The BTSCC results found in the present study are also noticeably higher compared to those of the U.S. (Schukken et al., 2003), the Netherlands (Santman-Berends et al., 2016) and Canada (Aghamohammadi et al., 2018). In Finland, a mastitis control program resulted in the reduction of BTSCC from 330000 (in 1988) to 170000 (in 1995) cells/mL within seven years (Honkanen-Buzalski and Myllys, 1996).

The overall SCM prevalence in Greece (increased from 2016 to 2018, 34.5% in 2018) was higher compared to countries like the Netherlands, Canada, England and Wales (Table 5), affecting one in three dairy cows every year. Furthermore, in 2018, heifers' SCM prevalence (23.4%) in Greece was 5% higher than in 2015, comparatively lower than the Netherlands and Belgium and higher than Spain, Sweden and Switzerland. However, it is important to consider that in all of these studies the SCC cut-offs used to define subclinical mastitis were lower than the cut-off used in the "IΩ" system. The lower cut-off could be responsible for the higher SCM prevalence observed in heifers of the Netherlands and Belgium, compared to Greece.

Average age at first calving was above the upper optimal threshold in all four years of the study (Table 1). Belated first calving has been associated with increased first lactation SCC and lower lifetime milk production, as well as longer calving intervals (also observed in the present study) and worse reproductive performance (Eastham et al., 2018).

In all four years of our study, the highest SCM incidence rate was observed in early lactation, accompanied by elevated SCS and SCM prevalence (Table 4). Many cows were probably already infected before or became infected at calving, implying a high possibility of either ineffective dry cow therapy or errors in dry and fresh cows' management. Dry period is a critical time in the lactation cycle (Bradley et al., 2010), as it is the optimum time to cure existing intramammary infections (Wilson et al., 1972) and a high-risk period for new intramammary infections (Smith et al., 1985). The probability of cows to develop new intramammary infections during the dry period has been related with high milk yield before drying-off, longer duration of the dry period, housing of dry cows in tie-stall barns, as well as with number of parity and SCS above 4.0 on last test-day before drying-off (Dingwell et al., 2002, Madouasse et al., 2012). Research has shown that cows from herds with high chances of maintaining or having newly elevated SCC over the dry period in the previous year had a higher probability of elevated SCC at first recording after calving (Madouasse et al., 2012).

The risk of new intramammary infections during the dry period is elevated during: i) the first weeks after drying-off, when involution of the udder occurs (Neave et al., 1950), and ii) the weeks preceding calving, when colostrogenesis takes place in the udder (Oliver et al., 1983). Therapeutic levels of antibiotics for dry cow therapy may be achieved only for the first 14 to 28 days after infusion, thus, failing to protect the udder during the last trimester of the dry period (especially for long ones); this can lead to new quarter intramammary infections during the dry period, which rate can reach 17.44% (Rindsig et al., 1978; Robert et al., 2006; Petzer et al., 2009). As a result, at the end of the dry period untreated and treated cows would stay at the same risk of new intramammary infections (Robert et al., 2006). In Greece, although not supported by data due to the retrospective nature of the study, it can be presumed that unsuccessful dry cow therapy and/or dry cow management errors could be involved into the current problem of high SCM incidence rate and SCM prevalence after calving and in early lactation. A focused research should be conducted to investigate the association between the applied dry period management practices in Greece and udder health.

Regarding heifers, the results indicated bad udder health from the very first lactation, even from the early stages (Tables 2, 3 and 4). Intramammary infections in dairy heifers may already occur at breeding age (Trinidad et al., 1990), but the risk is greater in the last trimester of pregnancy and at calving (Fox et al., 1995; Piepers et al., 2009). Heifers' intramammary infections during (late) gestation and early lactation are crucial not only for first lactation, but also for future milk production. That is because they are associated with impaired development of the mammary gland, negatively affecting both first lactation and future udder health, while can additionally lead to elevated risk of culling in first lactation (De Vliegher et al., 2005; Piepers et al., 2009; Santman-Berends et al., 2012). First lactation somatic cell count (especially in ear-

ly lactation) is negatively associated with both first lactation and lifetime milk yield (De Vliegher et al., 2005; Archer et al., 2014). Subclinical mastitis on first test-day after calving led to a higher risk of developing chronic mastitis or early culling and, moreover, isolation of major pathogens from first calving day up to the 5th day-in-milk was associated with 60% increased culling risk in first lactation (Compton et al., 2007; Bludau et al., 2014).

Apart from the negative effects of intramammary infections on the secretory tissue, suboptimal production has been associated with permanently elevated SCC throughout the whole lactation (De Vliegher et al., 2005). The longer the intramammary infections exist and the longer they persist into lactation (as observed in the present study by the elevated chronic SCM prevalence in first lactation), the larger the impact on heifers' production potential (Piepers et al., 2009). Given the fact that prevention rather than cure of early lactation elevated SCC is needed (De Vliegher et al., 2005), a series of preventive interventions are crucial for the improvement of the potential dairy herd productivity.

## CONCLUSION

The results of this study indicated high prevalence of SCM and poor udder health in Greek Holstein dairy herds, leading to shorter productive life and lower milk quality (elevated somatic cell count). Moreover, the average 305-days milk yield of 3rd lactation was lower than 2nd lactation cows, in 46% of the herds. These findings underline the necessity of implementing a series of actions in order to control subclinical mastitis among Greek dairy herds. The investigation of the applied herd management practices, accompanied by the development of a national mastitis monitoring and prevention program is considered important.

## CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to declare.

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Research article
Ερευνητικό άρθρο

# Effect of seasonal infertility period on boar sperm proteins and quality characteristics

**I.A. Michos[1], I.A. Tsakmakidis[1*], M. Tsantarliotou[1], G. Tsousis[1], P. Hadweh[2], C.M. Boscos[1]**

[1]*School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

[2]*Shool of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

**ABSTRACT.** Swine seasonal infertility reduces the productivity and profitability of a pig farm. The main causes of this condition are elevated environmental temperatures and long photoperiod during the summer season. The aim of this study was to investigate which sperm proteins and parameters are affected during the period of seasonal infertility. Depending on the environmental temperatures, the period from October to June was considered as cold and the period from July to September as warm season. A total of 65 ejaculates from 18 boars were collected over a year. Each semen sample was evaluated for kinetics (Computer Assisted Semen Analyzer), morphology (Sperm Blue stain), viability (Propidium Iodide - Calcein AM stain), mitochondrial membrane potential (Rhodamine 123 – Propidium Iodide stain), membrane integrity and functionality (Hypo-osmotic swelling test) and sperm DNA integrity (Acridine Orange Test). Moreover, selected proteins (HSP90, GPX5, OPN) were detected and quantified. The kinetic parameters VSL, LIN and the midpiece abnormalities were significantly higher in the warm compared to the cold season ($p<0.05$), while a strong tendency towards higher values for HSP90 and GPX5 was observed in warm compared to cold season ($p=0.07$ and $p=0.06$, respectively). In conclusion, among the boar sperm characteristics tested in our study, seasonal infertility period negatively affected VSL and LIN kinetics, while GPX5 seminal plasma enzyme and HSP90 sperm surface protein increased their sperm protective effects.

***Keywords*:** boar semen, semen analysis, HSP90, GPX5, seasonal infertility

*Corresponding Author:*
Ioannis A. Tsakmakidis, School of Veterinary Medicine, Faculty of Health Sciences, Farm Animals Clinic, St. Voutyra 11, 546 27, Thessaloniki
Aristotle University of Thessaloniki, Greece
E-mail address: iat@vet.auth.gr

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## INTRODUCTION

Seasonal differences in pig productivity have been repeatedly reported, particularly in geographical areas with tropical and hot climate (Peña et al., 2016). The summer is usually characterized as the season of infertility in swine industry. It is accompanied by anestrus or low expression and abnormal duration of estrus, prolonged or abnormal weaning to estrus intervals, high return to estrus rates, low pregnancy and farrowing rates, smaller litter sizes and lower boar fertilizing ability (Peltoniemi et al., 1999; De Rensis and Kirkwood, 2016).

Seasonal infertility has been attributed to heat stress, photoperiod, humidity, genetic background and management systems (De Rensis et al., 2017). Although some researchers suggest that heat stress is the main cause of this condition (Prunier et al., 1994), others indicate the photoperiod as the most important factor. Flowers (1997, 2015) found significant semen degradation when boars were kept at 34° C for 8-16 hours daily for 11 weeks or at 26-29° C for 10-14 weeks. However, Peltoniemi et al. (1999) reported seasonal infertility effects in Finland at ambient temperature that did not exceeded 25° C, thus implying the photoperiod as a more important cause.

Regarding the effects of heat stress on boar sperm, most studies investigated basic semen parameters and reported lower volume and concentration, lower sperm motility and higher percentage of morphological abnormalities during warm periods (Egbunike and Dede 1980; Barranco et al., 2013). Peña et al. (2019) found that tropical summer induces boar sperm DNA damage; however, no further studies have investigated effects of seasonality on specific, functional semen parameters.

Seminal plasma proteins are involved in spermatozoa's motion, capacitation and stress protection, thus influencing their function (Gonzalez-Gadavid et al., 2014). Killian et al. (1993) found osteopontin (OPN), a seminal plasma protein (55 kDA), to be associated with high fertility in bulls. In swine IVF the use of the OPN improved embryo development (Hao et al., 2008) and reduced polyspermy rates (Hao et al., 2006). Moreover, some membrane proteins play a crucial role to sperm regulation, communication and protection from oxidative stress (Strzezek, 2005). Among them, Heat Shock Protein (HSP) group helps the cells withstand extreme temperature variations. HSP90 is the most noted sperm surface protein. Its low concentration has been correlated to low quality boar sperm during warm periods (Huang et al., 2000; Valencia et al., 2017). Furthermore, it is well known that boar spermatozoa are susceptible to oxidative damage due to their relative high content of unsaturated fatty acids. Glutathione peroxidase-5 (GPX5) is an $H_2O_2$-scavenging enzyme identified in boar seminal plasma. GPX5 prevents premature capacitation. It is anchored on sperm plasma membrane or free into the epididymal fluid (Drevet, 2006). Vilagran et al. (2016) found that GPX5 of seminal plasma is positive related to sperm quality and Barranco et al. (2016) found a positive correlation between sperm quality and fertilization outcome after artificial insemination with liquid-stored boar semen.

Thus, the aim of the present study was to investigate which of the boar sperm parameters and proteins are affected by seasonality. Throughout a whole year, under commercial pig farming conditions, we evaluated the changes of semen analysis parameters and sperm proteins in order to detect changes between the seasonal infertility (warm) and normal productivity (cold) periods.

## MATERIALS AND METHODS

All reagents and chemicals used in this study were purchased from Sigma-Aldrich, (St Louis, MO, USA), unless otherwise specified.

### Animals, management and data recording

The study was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece and all operations were carried out according to the University's Guidelines for Animal Research.

As cold season was considered the period of semen collections between October and June (mean temperature 12.5° C), while as warm season was considered the period between July and September (mean temperature 23.9° C).

A healthy population of 18 crossbred sexually matured boars (2-3 years old) was used as sperm donors. All boars were used for artificial insemination (AI) in routine basis twice a week with a 3-day interval between collections. A total of 65 ejaculates (3 ejaculates per boar at average) were collected and tested in this study, over a 12-month period. The animals were properly housed in a commercial pig farm (Imathia region, Greece, 40°36'52.9"N, 22°22'08.1"E). Water was provided *ad libitum*, and animals were fed according to the standard nutrition protocols for adult boars.

**Semen collection, separation of seminal plasma and spermatozoa**

Sperm-rich ejaculate fractions were collected once per week using the gloved-hand technique. The sperm rich fraction was filtered through gauze and divided into aliquots. The first aliquot was extended with a commercial extender (M III®, Minitube, Germany) to a final concentration of $3x10^7$ spermatozoa/ml, divided to doses ready for insemination and stored at 17° C. Later a dose was transported to the laboratory (within an hour), inside an air-conditioned isothermal box (Minitube, Germany) adjusted at 17° C for further analysis, while the remaining doses were used for AI.

A second aliquot of the collected semen was used for the assessment of sperm and seminal plasma proteins. Semen was immediately mixed with a protease inhibitor cocktail [4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin] as proposed by González-Cadavid et al. (2014). Then, it was centrifuged at 640 x g for 15 minutes at 17° C to separate sperm and seminal plasma. Seminal plasma was centrifuged once more at 10,000 x g for 15 min at 17° C and supernatant was finally stored at -80° C until further examination.

Sperm pellet was washed with Phosphate Buffer Solution (PBS) and diluted at 1.5 x $10^9$ spermatozoa per ml (determined photometrically). According to Vilagran et al. (2013), sperm was once again pelleted at 640 x g for 3 minutes at 17° C, washed with 10mL PBS and re -centrifuged at 800 x g for 5 minutes at 17°C. Pellets were resuspended with HAM F-10 1X (Thermofisher® Scientific, USA) and samples were once again centrifuged and supernatant was discarded. Spermatozoa were solubilized in NP-40 lysis buffer [50 mM Tris–HCL pH 7.4, 250 mM NaCl, 5 mM EDTA, 1% Glycerol, 0.5% NP-40, 1 mM DTT, 1 mM PMSF 100 mM, 1× protease inhibitor cocktail (Roche)] and the sample was stored at -80° C until further analysis.

**Semen evaluation**

Upon the arrival at the laboratory, each sperm sample was warmed at 37 °C and a Computer Assisted Sperm Analyzer (CASA) was used (Sperm Class Analyzer®, Microptic S.L., Barcelona, Spain) to assess motility parameters such as sperm total motility (TM, %), progressive motility (PM, %), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, μm), straightness (STR, VSL/VAP, %), linearity (LIN, VSL/VCL, %), wobble (WOB, VAP/VCL, %), rapid, medium and slow moving spermatozoa (10<slow<25<medium<45< rapid μm/sec), and hyperactivation (sperm subpopulation of increased VCL> 97 μm/sec, ALH>3.5μm and LIN <0.32%). CASA configuration was set at 25 frames/sec, region of particle control 10-18 microns, depth of field 10 microns, progressive movement of > 45% of the indicator STR, circumferential movement <50% LIN (Karageorgiou et al., 2016). An aliquot of sperm sample (10μl) was placed on a pre-warmed Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and the evaluation was performed according to manufacturer's instructions. Pictures of at least 5 random fields were taken (x10), with a minimum of 1000 analyzed spermatozoa. Sperm evaluation was performed by a phase-contrast microscope with a thermal plate attached (Zeiss, Axio, Scope A1. Germany) and consecutive images were obtained by digital camera (Olympus BX 41, Japan), digitized and analyzed with Sperm Class Analyzer® software.

Morphology was estimated by Spermblue® staining method (Microptic S.L., Barcelona, Spain) according to manufacturer's instructions. Magnification x400 was used and 200 spermatozoa per slide were estimated and counted while results were expressed in % ratio. Spermatozoa were classified as normal and abnormal. As abnormal spermatozoa were considered those with morphological abnormalities such as abnormal heads, midpieces, tails and proximal or distal cytoplasmic droplets. Spermatozoa with abnormal acrosomes or detached heads were also classified as abnormal.

The viability was assessed by Propidium Iodide (PI; 0.75 mmol/L) - Calcein AM (1 mmol/L) double staining method (Basioura et al., 2018). A total of 200 spermatozoa were scored and results were expressed in % ratio.

According to Najafi (2013), Rhodamine 123 (Rh123) and PI dual fluorescent staining is used to provide critical information about mitochondrial membrane potential. Digital photos were instantly taken under fluorescent microscope and a total of 200 spermatozoa were scored. The percentage of sperm with functional mitochondria was identified by R123 high fluorescence and no PI fluorescence and results were expressed in % ratio.

Hypo-osmotic swelling test (HOST) was performed to evaluate sperm membranes' biochemical

activity and functionality. Aliquots of each semen sample (0.1 ml) were added to 0.9 ml of the hypotonic solution (150 mOsm/L) and incubated at 37° C for 60 min. A wet smear was made and left to air dry. Later the sample was examined under a phase contrast microscope at x400 magnification. A total of 200 spermatozoa was examined and categorized as positive or negative and results were expressed in % ratio. As positive spermatozoon with intact membranes was considered that with a swelling at the flagellum or was curled at any point. As negative were considered spermatozoa without no morphological alterations after the subjection of hypo-osmotic swelling test. Results were expressed in % ratio and a total of 200 spermatozoa were scored.

Acridine orange test (AOT) was used to estimate sperm DNA integrity as described by Tejada et al. (1984). Spermatozoa with normal double-stranded DNA displayed green fluorescence, whereas denatured single stranded DNA displayed as yellow-orange to red fluorescence. A total of 200 spermatozoa were examined under fluorescent microscope and the results were expressed in % ratio.

**Seminal plasma and sperm protein extraction and quantification by Western Blot (WB)**

In order to assess spermatozoa and seminal plasma proteins, a frozen aliquot of each sample was thawed and the Bradford method (Bradford, 1976) was used to determine the total protein concentration using a spectrophotometer (Quick Start™ Bradford Protein Assay; Bio-rad), and bovine albumin was used for the construction of the standard curve.

The sperm pellet was washed three times with PBS and centrifuged at 800 x g for 1 minute. Pellets were lysed in 5xSDS loading buffer (Tris 250mM, SDS 10%, Glycerol 50%, β-Mercaptoethanol 15%), boiled for 5 minutes and equal amount of protein lysates were loaded onto 12% slab gels for electrophoresis (SDS-PAGE). Electrophoresis was performed using a Mini Protean 3 Cell apparatus (Bio-Rad, Berkley, CA) with 50 mA/gel constant current for 1 h.

The proteins were then transferred to nitrocellulose membranes (GE Healthcare) under 50mA/300V for 1h (PowerPac 1000, Bio-Rad). Following this procedure, membrane was washed three times with TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween; pH 7.5) and incubated with blocking solution (5% skimmed milk powder in PBST) for 1 h. Afterwards, the membrane was washed for 5 minutes in TBST and then incubated for 2 h with the following primary antibodies: rabbit polyclonal anti-HSP 90 (AP-22747PU-N; Acris; diluted 1:1000 with TTBS), rabbit polyclonal anti-GPX5 (18731-1-AP; Proteintech Europe; diluted 1:500) and rabbit polyclonal anti- beta actin (ab8227, Abcam). Membranes were washed 3 times and subsequently incubated with a horseradish peroxidase-conjugated polyclonal goat anti-rabbit immunoglobin (SC-2004; Santa Cruz; 1:2000). Pierce™ ECL Plus Western Blotting Substrate (Thermoscientific, IL, USA) was used for development and membranes were scanned with Typhoon FLA 7000 (GE Healthcare). Protein levels were expressed as "band volume". That is the total signal intensity measured inside the boundary of a band in pixel intensity units. Protein bands were quantified using ImageJ (v.1.8.0), with actin serving as a normalizing factor. Their ratio was given as the level of proteins, according to Vilagran et al. (2013). The mean of three different measurements for each sample was taken into account.

According to previously published data (Valencia et al., 2017), SDS-PAGE, Western Blot and quantification of seminal plasma proteins were likewise carried out. Anti-OPN (GTX 37582; GeneTextech; diluted 1:200) was used for the detection of osteopontin while a non-specific band from Ponceau S staining was used as a normalizing factor. Both bands were quantified with ImageJ (v.1.8.0) and their ratio was used for graphical presentation. The mean of three different measurements for each seminal plasma sample was taken into account.

**Statistical analysis**

Statistical analysis was conducted using the Statistical Analysis System V9.3 (SAS Institute Inc., Cary, NC, USA). The Shapiro–Wilk test was performed in all outcome variables to test for the underlying distribution of the data. The distribution of total motility, VCL, ALH, hyperactivation, head, midpiece and tail abnormality cytoplasmatic droplet, HSP90, GPX5, OPN70 and OPN12 was different from normal distribution; thus, a Wilcoxon's two sample non-parametric tests was applied. All other parameters showed a normal distribution. For these variables a two-sample t-test for independent observations was used. Data are presented as mean ± standard deviation for data with normal distribution and as median ± median absolute deviation (MAD) for data not normally distributed. All analyses were considered to be statistically significant at $P < 0.05$ and tended to differ if $0.10 > P \geq 0.05$.

## RESULTS

The results of motility and kinetics in the two seasons are presented in table 1. Significant lower values of VSL and LIN were noticed in warm compared to cold season (p=0.04 and p=0.03, respectively), while the remaining parameters were not significantly different (p>0.05).

**Table 1.** Boar sperm motility and kinetics after CASA analysis during warm and cold season (mean or median ± standard deviation or MAD)

| Variable | Warm season (n=11) | Cold season (n=54) | p-value |
|---|---|---|---|
| Total motility (%) | 88.60 ± 3.29 | 90.79 ± 5.73 | 0.23 |
| Non progressive motility (%) | 39.26 ± 9.01 | 40.06 ± 10.68 | 0.82 |
| Progressive motility (%) | 47.58 ± 13.15 | 48.88 ± 15.12 | 0.79 |
| Rapid moving spermatozoa (%) | 48.19 ± 17.59 | 51.78 ± 22.58 | 0.62 |
| Medium moving spermatozoa (%) | 21.05 ± 6.56 | 19.45 ± 9.39 | 0.59 |
| Slow moving spermatozoa (%) | 17.61 ± 7.85 | 17.70 ± 10.42 | 0.98 |
| VCL | 56.34 ± 12.90 | 55.02 ± 12.26 | 0.83 |
| VSL | 19.26 ± 6.20 | 23.91 ± 7.54 | 0.04 |
| VAP | 33.91 ± 9.11 | 41.09 ± 12.53 | 0.08 |
| LIN | 35.47 ± 4.39 | 40.45 ± 12.81 | 0.03 |
| STR | 56.56 ± 7.79 | 58.59 ± 9.99 | 0.53 |
| WOB | 63.22 ± 7.04 | 68.36 ± 14.36 | 0.09 |
| ALH | 2.02 ± 0.13 | 2.11 ± 0.28 | 0.19 |
| BCF | 9.68 ± 2.91 | 9.37 ± 4.23 | 0.82 |
| Hyperactivated spermatozoa (%) | 0.97 ± 0.24 | 1.59 ± 0.15 | 0.19 |

Abbreviations: VCL: curvilinear velocity (μm/sec); VSL: straight line velocity (μm/sec); VAP: average path velocity (μm/sec); LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz).

No significant differences were observed in boar sperm morphological and functional characteristics between seasons (p>0.05), except for midpiece abnormalities which were higher in the warm period (p=0.01, table 2).

**Table 2.** Boar sperm morphological and functional characteristics during warm and cold season (mean or median ± standard deviation or MAD)

| Variable | Warm season (n=11) | Cold season (n=54) | p-value |
|---|---|---|---|
| Normal morphology (%) | 75.59 ± 12.84 | 76.06 ± 9.92 | 0.89 |
| Head abnormalities (%) | 9.5 ± 4.5 | 6.5 ± 3 | 0.68 |
| Midpiece abnormalities (%) | 2.0 ± 1.0 | 1.0 ± 0.5 | 0.01 |
| Tail abnormalities (%) | 9.0 ± 6.0 | 12.5 ± 6.0 | 0.33 |
| Cytoplasmic droplets (%) | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.92 |
| Viability (%) | 81.86 ± 5.84 | 83.01 ± 8.08 | 0.66 |
| HOST+ spermatozoa (%) | 40.14 ± 13.59 | 38.61 ± 12.37 | 0.72 |
| Activated Mitochondria (%) | 86.50 ± 3.97 | 84.49 ± 7.90 | 0.52 |
| Sperm DNA damaged (%) | 1.9 ± 0.34 | 1.6 ± 0.33 | 0.79 |

No significant differences were found in sperm protein quantities (p>0.05, table 3). However, a strong tendency towards higher values of HSP90 and GPX5 in warm compared to cold period was found (p=0.07 and p=0.06, respectively).

**Table 3.** Boar sperm and seminal plasma proteins during warm and cold season (median ± MAD)

| Variable | Warm season (n=11) | Cold season (n=54) | p-value |
|---|---|---|---|
| HSP90 | 1.58 ± 1.27 | 1.06 ± 0.80 | 0.07 |
| GPX5 | 3.59 ± 1.38 | 1.98 ± 1.39 | 0.06 |
| OPN70 | 0.60 ± 0.59 | 1.95 ± 1.1 | 0.19 |
| OPN12 | 2.38 ± 2.37 | 2.01 ± 1.97 | 0.81 |

## DISCUSSION

Seasonal infertility in swine reduces significantly the productivity of a pig farm. This is mainly the result of the decrease in reproductive parameters, i.e. lower farrowing rates, lower number of live born piglets and decreased litter sizes (Love, 1978; Peltoniemi et al. 1999). Females and males are both affected by seasonal infertility. Concerning the boar, previous studies reported that heat stress results in reduced libido and decreased semen volume and concentration (Cameron and Blackshaw, 1980; Flowers, 1997).

The present study found seasonal differences of semen parameters which can be contributed to the outcome of AI. Among kinetics, VSL and LIN were lower in warm compared to cold period. It is known that good sperm kinetics can positively affect fertility. These results can influence the seasonal reproductive differences in a farm, since VSL is correlated with the total number of live born piglets (Broekhuijse et al., 2012). Lee et al. (2014) proposed that VSL should be considered to select semen for AI. Although LIN is not a sensitive indicator of sperm motion, Hirai et al. (2001) found a higher non-return to estrus rate in boars with significantly higher LIN. Additionally, Casas et al. (2009), reported that a combination of LIN and STR can be advantageous for the freezability of boar sperm. Moreover, it is well known that sperm motility is one of the most important indicators of field fertilizing ability. It correlates with litter size in pigs, even though an AI with a low number of spermatozoa per dose takes place (Tardif et al., 1999). In the present study no significant differences between warm and cold season were found regarding total and progressive motility. This finding agrees with the study of Popwell and Flowers (2004), who monitored three boars' semen quality with significantly different *in vitro* and *in vivo* fertility for 40 weeks, but they did not reveal total motility differences. Although most of the published studies agree that motility and kinetics play a crucial role in the fertilization process, there are reports that show different results. Reproductive efficiency can be attributed to other important factors, such as boar genetics, farm management, enriched nutrition and animals' care. Moreover, the results of the kinetics CASA measurements can be affected by many factors, i.e. the sample preincubation time, the different sperm counting chamber, the software settings and the user training (Yeste et al., 2018). Therefore, only the results which emerged following similar methodology can be compared.

In our study no effect of season on sperm quality and functional characteristics was noticed, with the exception of the percentage of midpiece abnormalities, indicating that motility parameters are more susceptible. Midpiece abnormalities have been related to Reactive Oxygen Species (ROS) generation, lipid peroxidation and increase in creatine kinase (CK) activity (Huszar and Vigue, 1994). CK, which is a marker of sperm maturity, has been related to low sperm fertilizing capacity (Hallak et al., 2001), while ROS hyperproduction and oxidative damage impair the normal function of spermatozoa (Radomil et al., 2011). Heat stress has also been associated with testicular dysfunction and oxidative stress (Hamilton et al., 2016). The determination of oxidative parameters was not within the scopes of the present study. However, GPX5 and HSP90 were assessed, demonstrating as a tendency higher values in warm compared to cold season. Hydrogen peroxide ($H_2O_2$) is considered as the major damaging ROS for boar sperm, while Glutathione Peroxidases (GPXs) are the responsible enzymes that neutralize $H_2O_2$ (Awda et al., 2009). Moreover, GPX5 protects boar spermatozoa during their route into the female genital tract, decreasing the harmful effects of ROS generated by uterine tissues. According to Barranco et al. (2016), GPX5 affects fertility after AI with liquid-stored boar semen. Based on the findings of our study, it is probable that boar spermatozoa exhibit higher GPX5 to overcome the extended ROS production during the periods of seasonal infertility. Additionally, HSP90 is the most noted sperm surface protein (Valencia et al., 2017) and it is reduced when sperm quality is low (Huang et al., 2000). In accordance with GPX5 changes, HSP90 was also increased in the warm period of our study to support a defensive mechanism of boar spermatozoa against heat stress. Many genomic regions responsible for heat tolerance have been identified in pigs (Riquet et al., 2017) reflecting the boar genetic improvement that has been succeeded up to today.

## CONCLUSION

In conclusion, among the boar sperm characteristics tested in our study, seasonal infertility period negatively affected VSL and LIN kinetics, while GPX5 seminal plasma enzyme and HSP90 sperm surface protein increased their sperm protective effects.

## ACKNOWLEDGEMENTS

The authors would like to thank the staff of the pig farm Karanikas LTD for their valuable help to the completion of this study.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

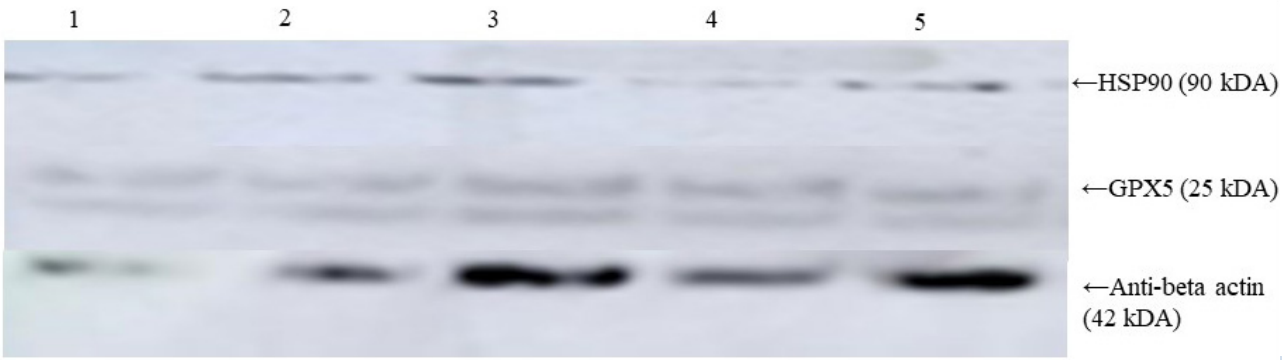


**Fig 1.** Representative band patterns from WB analysis of HSP90 and GPX5 from the solubilized membranous fraction of boar spermatozoa

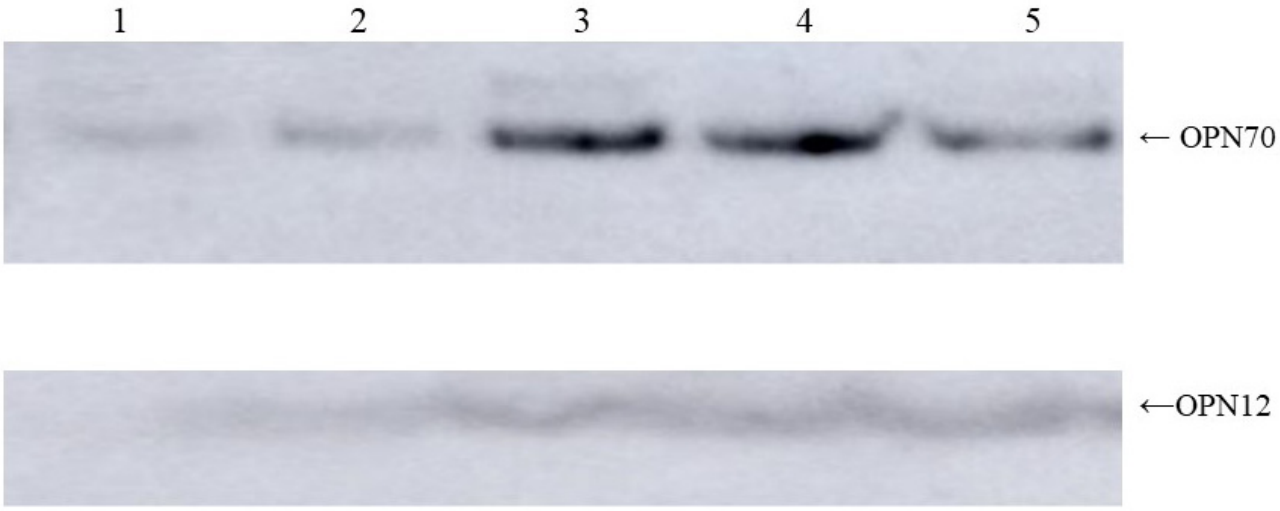


**Fig 2.** Representative band patterns from WB analysis of OPN from the boar seminal plasma

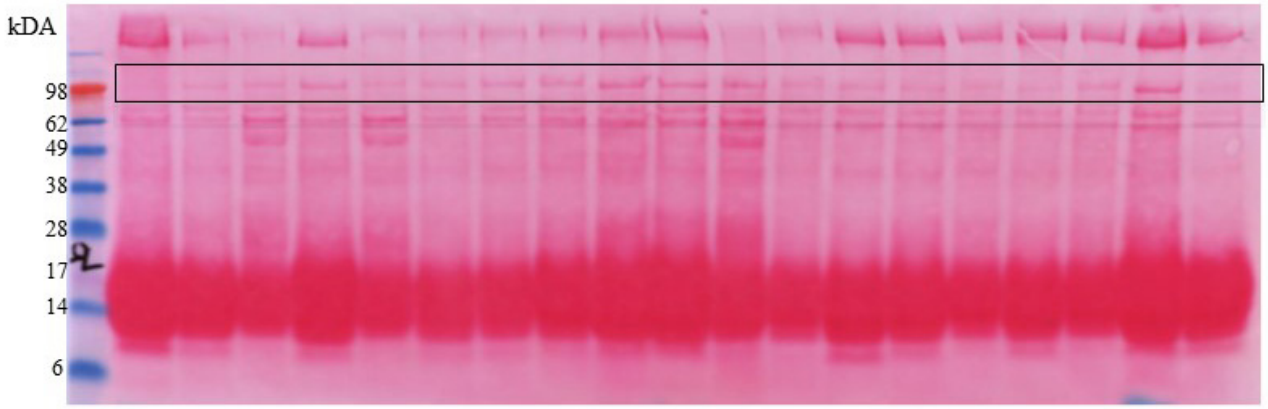


**Fig 3.** Nitrocellulose blot stained with Ponceau S. The protein band used as OPN control is highlighted

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Research article
Ερευνητικό άρθρο

# Unilateral Spinal Anaesthesia in Calves

**S. Yayla[a], E. Kilic*, U. Aydin*, I. Ozaydin*, V. Baran*, A. Kamiloglu***

[a] *Department of Surgery, Faculty of Veterinary Medicine, University of Dicle, Diyarbakır, Turkey*

* *Department of Surgery, Faculty of Veterinary Medicine, University of Kafkas, Kars, Turkey*

**ABSTRACT:** In this study, we aimed to evaluate the effects of unilateral anaesthesia by the administration of hyperbaric bupivacaine through the lumbosacral space into the subarachnoid space in calves. A total of 10 calves with unilateral femoral fractures were included in the study. After each calf was placed in a lateral position on the side intended for surgery, 15 mg of hyperbaric bupivacaine was slowly injected into the subarachnoid space. The onset, duration and depth of anaesthesia were determined by the pinprick test (scale 1–4). In addition, heart rate, diastolic arterial blood pressure, systolic arterial blood pressure, mean arterial blood pressure, respiratory rate and body temperature of the calves were monitored and recorded from the onset to 120 min after anaesthesia. The onset of unilateral spinal anaesthesia was within 20 s and the mean duration of anaesthesia was 155.40 min. Although there were statistical differences between hemodynamic values in the study, they were within the reference values. As a result, we believe that unilateral spinal anaesthesia in calves provides adequate anaesthesia for use in orthopaedic procedures; thus, it can be used in practice.

***Keywords:*** Unilateral spinal anaesthesia, hyperbaric bupivacaine, calves

*Corresponding Author:*
Sadik Yayla, Department of Surgery, Faculty of Veterinary Medicine, University of Dicle, Diyarbakır, Turkey, 21280
E-mail address: sadikyayla@gmail.com

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## INTRODUCTION

The blockage of sympathetic, sensory and motor nervous fibers rapidly occurs after administration of a local anaesthetic into the subarachnoid space and this procedure is referred to as subarachnoid, spinal or intrathecal anaesthesia (Derossi et al., 2007; Ozaydin and Kilic, 2003; Yayla and Kilic, 2010;Yayla et al., 2013). This technique can be performed in small ruminants and calves by injecting local anaesthetics into the subdural space through the lumbosacral junction (Derossi et al., 2007; Yayla et al., 2013). Spinal (intrathecal) anaesthesia provides appropriate surgical conditions for caesarean section, abdominal, pelvic or hind leg operations (Buttner et al., 2016; Malinovsky et al., 2000; Yayla et al., 2013).

Bupivacaine, commonly used in spinal anaesthesia, is an amide-type long-acting local anaesthetic. Although several side effects of bupivacaine such as ventricular arrhythmia and cardio toxicity have been reported in humans, it still remains popular (Liu and Lin, 2009; Malinovsky et al., 2000).

In spinal anaesthesia, factors such as dose of the local anaesthetic agent, injection site, additives in the anaesthetic drug, pH, baricity and temperature of the anaesthetic agents are influential on the local anaesthetic activity and its spread in the subarachnoid space (Yayla and Kilic, 2010;Yayla et al., 2013). Hypobaric solutions have specific gravity less than that of the cerebrospinal fluid (CSF), and can cranially migrate following injection and affect the diaphragm and intercostal muscles, which may cause death. In contrast, because hyperbaric solutions are heavier than CSF, they are locally retained or show limited spread in the injection site (Yayla and Kilic, 2010). Therefore, the use of hyperbaric local anaesthetic solutions may be an attractive option to avoid potential complications (Skarda and Tranquilli 2007; Yayla et al., 2013).

In humans, when a hyperbaric local anaesthetic is intrathecally administered, the anaesthetic result that is achieved by introducing the effect of the local anaesthetic on nerve roots in a certain area by limiting the distribution of the local anaesthetic with the effect of gravity and patient's position is defined as unilateral spinal anaesthesia (Kilavuz et al., 2015). However, this technique has not been used in the field of veterinary medicine. The hypothesis of this study is that hyperbaric local anaesthetic agent acts unilaterally on the spinal cord with the effect of gravity. In this study, we aimed to evaluate the effects of unilateral anaesthesia provided by the administration of hyperbaric bupivacaine solution through the lumbosacral space into the subarachnoid space in calves.

## MATERIALS AND METHODS

The study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK 2018/006). Ten calves with unilateral femoral fractures were included in the study. Unilateral femoral fracture was sufficient without any criteria for selection of these calves. Osteosynthesis with intramedullary nail was decided in all calves included in the study.

Following routine preparations such as clipping and disinfecting, the calves were sedated with xylazine (2% Rompun®, Bayer, Turkey, 0.2 mg/kg intramuscular) and placed on the operating table in a lateral position on the side intended for surgery. The operation table was tilted at approximately 30°, with the head and chest region of the animal facing upward. Under aseptic conditions, skin and subcutaneous tissues in the lumbosacral region were desensitized with 2 ml of the local anaesthetic (Adokaine®, Sanovel, Turkey) for subcutaneous tissue and ligamentum flavum. An 18 G spinal needle was used to enter the subarachnoid space through the lumbosacral junction (L6-S1) and the needle placement was confirmed by observing the flow of the CSF. Subsequently, the injection of Marcaine® Spinal Heavy 0.5% (Astra Zenaca) containing 15 mg/total (3 ml) of hyperbaric bupivacaine was slowly performed. All injections were performed by the same operator (first author, SY). After keeping the calves in this position for 15 min, they were again placed in a suitable position and osteosynthesis was performed. In addition, an electrolyte solution was intravenously administered in the jugular vein (0.9% saline) at the rate of 10 ml/kg/h throughout the operation.

Vital signs of each animal used in the study were monitored (Veterinary Monitor® MMED6000DP S6-V). Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), heart rate (HR), respiratory rate (RR) and rectal temperature (RT) values were recorded at the onset, during sedation, and at 5, 15, 30, 60, 90 and 120 min after spinal anaesthesia. The onset and duration of anaesthesia were determined by the pinprick test. Pin-prick test was performed for 180 minutes from the beginning of anaesthesia. The pinprick test was evaluated on a scale of 1–4 as described by DeRossi et al. (2007) and Yayla et al. (2013) (1, no analgesia and reaction

to stimulus; 2, mild analgesia and depressed reaction to stimulus; 3, moderate analgesia and no response to superficial needle-prick stimulation of the skin in response to stimulus and 4, complete analgesia and no response to insertion of the needle deep into the muscle layer). In addition, the anaesthetic effect was compared by performing this evaluation both on the leg intended for surgery and on the intact leg.

For postoperative pain, ketoprofen (Ketobay®, Bayer, Turkey, 3 mg/kg, subcutaneously) was administered for up to 3 days after surgery.

Statistical analysis of the data was performed using the Minitab-16 software package. The Anderson–Darling test was used to test the normality distribution of the data, a Kruskal–Wallis test was used for non-parametric data and one-way analysis of variance (ANOVA, Tukey's pairwise comparisons) with $p < 0.05$ was accepted as significant.

## RESULTS

All the calves included in the study (n = 10) belonged to the Simmental breed, seven were male and the remaining three were female. The calves were 1-month old with unilateral femoral fractures and their mean live weight was 43.30 ± 4.88 kg. The mean operation time for osteosynthesis was 47.50 ± 8.90 min.

Subarachnoid injections through the lumbosacral region were easily performed, and after these injections, the onset of anaesthesia was within 20 s in all cases. A comparative evaluation of both hind legs using the pinprick test and analgesia scores is summarized in Figure 1. There was a statistically significant difference between the dependent leg and the contralateral leg from the 1st minute after the lumbosacral injection ($P < 0.05$, Kruskal–Wallis test). Unilateral spinal or intrathecal anaesthesia was performed in all calves and the mean duration of anaesthesia was calculated as 155.40 ± 27.71 min.

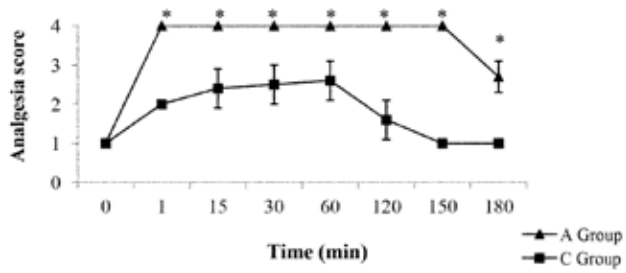


**Figure 1.** Analgesia scores in response to standard pinprick stimuli in calves that received unilateral spinal anaesthesia with hyperbaric bupivacaine. A group: hind leg affected by unilateral anaesthesia. C group: intact hind leg. (1, no analgesia; 2, mild analgesia; 3, moderate analgesia and 4, complete analgesia). *Statistically significant difference between the affected and unaffected legs ($P < 0.05$).

During the operation, both the depth of anaesthesia and muscle relaxation was deemed adequate for osteosynthesis and no further interventions were required.

Hemodynamic values obtained from the study (HR, RR, SBP, MBP, DBP and RT values) are summarized in Table 1. Although there was a statistically significant (ANOVA, One Way test) decrease from baseline in SBP value following the administration of xylazine, SBP increased as from the 30th to the 60th minute of anaesthesia. There was a statistically significant difference in the diastolic value from baseline, but improved after 60 min. In terms of MBP and HR values, there was a statistically significant between the initial and the 5th minute of spinal anaesthesia.

**Table 1.** Mean ± sd of heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), respiratory rate (RR) and rectal temperature (RT) in calves that received unilateral spinal anaesthesia with hyperbaric bupivacaine.

| Values | Time (min) | | | | | | | | P |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | Sedation | Spinal anaesthesia | | | | | | |
| | | | 5 | 15 | 30 | 60 | 90 | 120 | |
| **SBP** | 100.20±13.46[a] | 68.50±13.04[b] | 69.80±14.09[b] | 81.20±11.86[b] | 81.30±7.83[b] | 85.20±6.44[ab] | 87.30±7.96[ab] | 99.10±12.49[a] | 0.000 |
| **DBP** | 80.80 ±7.47[a] | 55.70 ±7.56[b] | 52.20 ±3.22[b] | 58.40±6.33[bc] | 66.60±6.96[c] | 78.80±7.02[a] | 81.30±7.47[a] | 82.40±7.34[a] | 0.000 |
| **MBP** | 90.10 ±18.27[a] | 69.20±12.10[b] | 70.60±15.48[b] | 75.60±12.64[ab] | 81.70±8.82[ab] | 82.40±15.56[ab] | 83.10±15.51[ab] | 88.50±11.73[a] | 0.008 |
| **HR** | 98.90 ±8.39[a] | 67.90±26.28[b] | 65.10±23.33[b] | 83.50±17.07[ab] | 91.80±12.77[a] | 99.70±9.53[a] | 99.60±8.17[a] | 100.60±8.95[a] | 0.000 |
| **RR** | 15.30 ±1.33[a] | 14.20 ±2.15[a] | 11.60 ±0.96[b] | 10.90±1.44[ab] | 12.50±1.08[a] | 13.90±1.44[a] | 14.40±1.43[a] | 15.20±0.63[a] | 0.000 |
| **RT** | 37.90 ±0.61[a] | 37.41±0.12[ab] | 37.29 ±0.29[b] | 37.40±0.22[b] | 37.57±0.25[ab] | 37.57±0.30[ab] | 37.60±0.26[ab] | 37.64±0.26[ab] | 0.004 |

a–c: Significantly different from baseline values on the same line ($p < 0.05$)

## DISCUSSION

Spinal or intrathecal anaesthesia is used in the fields of both human and veterinary medicine because of the several benefits it offers (Kilavuz et al., 2015; Ozaydin and Kilic, 2003; Singh et al., 2014; Yayla et al., 2013; Yayla et al., 2017). While unilateral spinal anaesthesia is widely used in human medicine (Esmaoglu et al., 1998; Kilavuz et al., 2015), its use has not been described in veterinary medicine or is not sufficiently known. Therefore, in this study, we aimed to evaluate the effects of unilateral anaesthesia provided by the administration of hyperbaric bupivacaine through the lumbosacral space to the subarachnoid space in calves. The data obtained from this study showed that unilateral spinal anaesthesia in calves provides adequate anaesthesia for use in orthopaedic procedures and can be used in practice.

There is a controversy over some side effects of spinal anaesthesia, particularly cardio respiratory effects (e.g. hypotension) of the local anaesthetics used in spinal anaesthesia, which is known to be a good alternative to general anaesthesia (Ozaydin and Kilic, 2003; Yayla and Kilic, 2010; Yayla et al., 2013). Utilization of unilateral spinal anaesthesia technique may have several advantages in orthopaedic procedures of the hind leg without these side effects (Buttner et al., 2016; Esmaoglu et al., 1998; Kilavuz et al., 2015; Singh et al., 2014). In unilateral spinal anaesthesia, use of low-dose local anaesthetics, slow injection, proper positioning of the patient, attention to baricity of the local anaesthetic used and maintenance of the appropriate position of the patient during injection are recommended for unilateral spread of the local anaesthetic drug (Kilavuz et al., 2015). The most important advantage of unilateral spinal anaesthesia compared with bilateral spinal anaesthesia is fewer and less severe cardiovascular side effects and a better nerve block on the side to be operated despite using a low dose. The maintenance of the position of the patient is a challenge (Esmaoglu et al., 1998; Kilavuz et al., 2015).

Spinal anaesthesia technique is based on the induction of spinal block by directly injecting the local anaesthetic into the cerebrospinal fluid (CSF), which surrounds the spinal cord, and nourishes and protects it in the medullar canal (Yayla and Kilic, 2010; Yayla et al., 2013). The local anaesthetic drug, which starts to spread over the spinal cord in the CSF, has an effect on the nerve roots exiting the spinal cord and dorsal root ganglia. In spinal anaesthesia, however, it is desirable to limit the spread of local anaesthesia in the CSF and to prevent local anaesthesia from spreading excessively cranially (Ozaydin and Kilic 2010; Yayla et al., 2013; Yayla et al., 2013). Several factors such as the injection rate, positioning of the patient, local anaesthetic baricity and patient's anatomy may affect the spread of the drug. In both bilateral and unilateral spinal anaesthesia, rapid injection is not recommended to avoid possible complications. In addition, because hyperbaric local anaesthetics are heavier than the CSF, they precipitate with the effect of gravity on the dependent part of the spinal cord and show their effect on the dependent nerves and nerve roots. Positioning the patient laterally is required. In addition, keeping the local anaesthetic dose low compared with that used in bilateral spinal anaesthesia contributes to the decrease in the volume of medication injected into the CSF and to a more limited spread (Casati et al., 1998; Fanelli et al., 2000; Kilavuz et al., 2015; Kuusniemi et al., 2000; Malinovsky et al., 2000; Yayla et al., 2013; Yayla et al., 2017). To the authors' knowledge, this is the first study on calves that examined hyperbaric bupivacaine as a local anaesthetic to provide unilateral spinal anaesthesia. Lumbosacral injection was performed in all animals by the same operator and at a very slow pace. The dose in calves was designed as 15 mg and 3 ml of anaesthetic drug was injected (Yayla et al., 2013). In addition, because the duration needed for the spread of the local anaesthetic in the CSF after injection or removal of the effect of the gravity has been reported to be 15 min (Yayla and Kilic, 2010; Kilavuz et al., 2015), calves were kept in a lateral position with the side intended for surgery dependent for at least 15 min following injection and were then positioned for surgery. In all the calves in this study, unilateral spinal anaesthesia was obtained within 20 s after injection and lasted for 155 min on average. Complete spinal block was not observed in the contralateral extremity (Figure 1).

Because of the slowing down of peripheral venous circulation in spinal anaesthesia, bradycardia or cardiovascular depression is an expected side effect. Studies have reported that despite being widely used in spinal anaesthesia, bupivacaine may trigger cardiopulmonary side effects (Casati et al., 1998; Fanelli et al., 2000; Kilavuz et al., 2015; Kuusniemi et al., 2000; Yayla et al., 2013). In our study, there was a significant decrease from baseline in SBP, DBP, MBP, HR and RR values after sedation. We attribute these changes to the effect of xylazine (Kamiloglu et al.,

2005). In addition, during spinal anaesthesia and especially after 15 min, xylazine was well tolerated in terms of values of hemodynamic parameters which returned to baseline and remained within the reference range. The depressive effect on hemodynamic parameters is closely related to the dose of the local anaesthetic used (Malinovsky et al., 2000). In this regard, it is important to use a low dose of a local anaesthetic in unilateral spinal anaesthesia. In fact, the most important advantage of unilateral spinal anaesthesia compared with bilateral spinal anaesthesia is the lower incidence of cardio respiratory side effects.

## CONCLUSION

In conclusion, unilateral spinal anaesthesia provided by the administration of intrathecal hyperbaric bupivacaine through the lumbosacral space with appropriate positioning in calves with unilateral femoral fractures provides adequate anaesthesia and of adequate duration to allow its use in orthopaedic procedures. We believe that unilateral spinal anaesthesia can be used in orthopaedic interventions for the hind leg in daily practice.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

# Immunological and pathological adverse effects of avian influenza virus subtype H9N2 infection in aflatoxicated-broiler chickens

**H.F. Ellakany[1], H.S. Abd-Elhamid[1], L.A. Tantawy[2], H.A. Elsamadony[2], A.R. Elbestawy[1*], A.R. Gado[1], Y.S. El-Sayed[3†], H.S. Ismael[2]**

[1] *Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Egypt*

[2] *Department of Poultry, Animal Health Research Institute, Dokki, Egypt*

[3] *Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhur University 22511, Egypt*

**ABSTRACT.** Aflatoxin B1 (AFB1) is a metabolic product of the *Aspergillus* spp. of molds, which grow on several feedstuffs stored in hot moist conditions. It is one of the immunosuppressive agents that might influence the pathogenesis of avian influenza virus (AIV) subtype H9N2 in broilers, which can exacerbate the disease outcomes. The immunological, biochemical and pathological adverse health effects of an interaction between low levels of dietary aflatoxins (AFs) and H9N2 infection in broiler chickens were investigated. One hundred and eighty of unvaccinated 1-day-old COBB chicks were, therefore, raised for 35 days in the following treatment groups: control, AFs, AFs+H9N2, and H9N2. AFs in the basal diet was added at 200 ppb starting from the first day of age, while H9N2 virus was intra-nasally installed at a dose of 100 µl of $10^6$ $EID_{50}$/bird of allantois fluid at 23rd day. Humoral and cell-mediated immune responses were evaluated. Evidence of H9N2-AIV viral shedding was also detected. It has been observed that concurrent exposure of AFs and H9N2 virus negatively affected chicken performance traits *i.e.* lowered feed intake and body weights with exaggerated respiratory and digestive disturbances, and 20% mortality rate. Ten days' post H9N2 infection, significant ($p \leq 0.05$) increment in serum transaminases (AST and ALT) and falling in cell-mediated immunity *i.e.* total leukocyte count, lymphocyte transformation activity and macrophage phagocytic activity were detected. Additionally, AFs+H9N2 significantly ($p \leq 0.05$) lowered H9N2-HI titers (5.5 Log2) than H9N2 alone (6.3 Log2). Pathologically, aflatoxicated chickens showed hydropic degeneration, hepatocytic vacuolation and necrosis of liver tissues with nephrosis and urates deposition in ureters, as well as bursal and thymic lesions, which were potent in H9N2–inoculated chickens. AFs exposure increased the incidence and titer of H9N2 viral shedding. It could be concluded that dietary contamination with AFs even at very low levels has explanatory effect in H9N2–inoculated broilers, and *vice versa*.

***Keywords***: Aflatoxins, AIV subtype H9N2, Immunological, Pathological, Broilers

*Corresponding Authors:*
A.R. Elbestawy, Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, Egypt.
E-mail address: ahmed.elbestawy@vetmed.dmu.edu.eg

Y.S. El-Sayed, Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University.
E-mail address: elsayed-ys@vetmed.dmu.edu.eg; yasser_tf@yahoo.com

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## INTRODUCTION

In developing countries, due to a lack of biosecurity, management practice and regulatory systems, free-range chickens are more regularly exposed to the risk of immunosuppressants like aflatoxins (AFs). Aflatoxins, difuranocoumarin compounds, are secondary toxic metabolites produced by toxigenic fungi; mostly *Aspergillus flavus* and *Aspergillus parasiticus* (Diener et al. 1987). Almost any feed, grains or their ingredients for poultry and livestock are able to favor fungal growth and AFs formation, causing health impairment and economic losses (Iheshiulor et al. 2011). Aflatoxin B1 (AFB1) is a potent hepato-toxicant causing acute liver damage; hepatocellular hyperplasia, necrosis, cirrhosis, and biliary hyperplasia in affected chickens. Secondary to the liver disease, the immunosuppressive activity of AFB1 is intimately linked to its toxicity (Yunus et al. 2011). It suppressed the development of bursa of Fabricius and thymus, reduced the weight and function of immune organs (Chen et al. 2014; Ellakany et al. 2011; Peng et al. 2015; Sur and Celik 2003), lowered the splenic T cell subsets percentages (Chen et al. 2014; Peng et al. 2014) and plasma cell counts (Celik et al. 2000), and arrest the B-cell cycle (Hu et al. 2018), immunoglobulin contents (He et al. 2014) as well as cytokines production (Jiang et al. 2015). Moreover, AFB1 induced oxidative stress and lipid peroxidation (Liu et al. 2016; Yuan et al. 2016), and mitochondria damage (Peng et al. 2016; Yuan et al. 2016) in the lymphoid organs. Aflatoxicosis, therefore, impairs humoral and cellular–mediated immune responses, which *in turn* increases the susceptibility of chicken to diseases *i.e.* avian influenza (AI) and infectious bursal disease (IBD), and causes vaccination failure (Bakshi et al. 2000; Gabal and Azzam 1998).

Avian influenza virus H9N2 is a low pathogenic panzootic pathogen that in spite of causing mild to moderate enteric and respiratory signs, it has been recently associated with high morbidity and considerable mortality with potential to infect human population (Ahad et al. 2013; Alexander 2003). H9N2 infection could decrease growth rate and feed conversion rate (FCR) in broilers due to co-infection with other pathogens as IBV, *Staphylococcus* exacerbates H9N2 influenza A virus infection in chickens (Kishida et al. 2004). Host cell-mediated immune response is important in the pathogenesis of avian influenza viruses and plays an important role in recovery from viral infection (Wells et al. 1981). As one of the immunosuppressive agents that influences the pathogenesis of H9N2 virus in broilers, aflatoxins, even in very low levels, can disturb the immune system of birds and thus can exacerbate the disease outcomes of H9N2-AIV (El Miniawy et al. 2014). In turkeys, aflatoxin delayed influenza virus clearance and lead to decreased IFN-γ mRNA expression and increased pathogenicity of H9N2 LPAI viruses under field conditions (Umar et al. 2015). Consequently, this work aimed to study the effect of H9N2-AIV virus co-infection with simultaneous aflatoxins addition in the feed of commercial broilers chickens.

## MATERIALS AND METHODS

### *Aspergillus flavus* strain and aflatoxin production

Standard aflatoxigenic *Asperigellus flavus* strain was obtained from the Department of Mycology, Animal Health Research Institute, Agriculture Research Center, Ministry of Agriculture, Egypt. Aflatoxins were produced by growing standard aflatoxigenic strains on sterile polished rice (West et al. 1973). Aflatoxins were detected quantitatively in rations by using affinity column chromatography (Aflatest 10, Naremco, Springfield, IL, USA) and fluorometry (Sequcia Tuner Model 450 with a 360 nm excitation filter and a 450 nm emission filter) (Nabney and Nesbitt 1965). All reagents were of the highest analytical grade.

### Challenge virus

Avian influenza A H9N2 virus strain (A/Chicken/Egypt/93/2015 with Genbank accession No: KY872759). This challenge strain was isolated from a broiler chicken flock and identified in the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Egypt (Abd El-Hamid et al. 2018).

### Animals experimentation and procedures

One hundred and eighty unvaccinated one-day-old healthy Cobb 500 broilers obtained from a commercial local hatchery and grown over a 35-d experimental period. The broiler chicks were floor-reared, weighed and randomly allocated with equalized initial body weights into four groups of three replicates (15 chick/replicate). (I) Control group; chicks received the AFs-free ration and served as a negative control. (II) AFs group; chicks received the AFs-containing basal diet. (III) AFs+H9N2 group; chicks received the AFs-containing diet and were inoculated with H9N2-AIV. (IV) H9N2 group; chicks received the control basal diet with no AFs but were inoculated with H9N2-AIV. The AFs-containing ration (200

ppb) was fed to chicks starting from the first day of age to the end of the experiment at 35 days. On the 23rd day of age, AIV-H9N2 inoculated groups were directly inoculated with 100 µl of $10^6$ $EID_{50}$ per bird of allantois fluid via intranasal route. Weekly, all birds were individually weighed starting from the 1st week until the end of the experiment. Performance traits including body weight as well as mortality, postmortem lesions, hematological and biochemical parameters, and pathological examinations were assessed.

The use of broilers and all mandatory laboratory health and safety experimental procedures involving animals had been complied by the Damanhour University Animal Care and Use Committee, Egypt. Nutritional requirements were adequate according to the National Research Council. All killed birds were euthanized by chloroform at a high dose to induce respiratory failure.

**Estimation of liver function indices**

At 10-day post-inoculation (dpi), twenty blood samples were collected from wing vein of chickens using appropriate sterile needles, syringes, and falcon tubes without anticoagulants. The samples were centrifuged at 1000× g for 10 min, and the sera were separated and then stored at −20°C until the assessment of liver function enzymes; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using supplied detection kits (BioMérieux, Lyon, France).

**Estimation of cell-mediated immune response**

Another heparinized blood samples were collected at 14, 27 (4 dpi) and 33 (10 dpi) days of age to evaluate the total and differential leukocyte counts (Jain 2000), lymphocyte transformation activity (Kumar and Das 1996) and macrophages phagocytic activity (Salaberria et al. 2013).

**Estimation of humoral-mediated immune response**

Hemagglutination inhibition (HI) test was done at day one of age according to the standard protocols of the World Health Organization (WHO 2002) for detection of maternal antibodies against H9N2-AIV, and then at 10 dpi for detection of H9N2 infection immune response. The HI titer (log2) was evaluated using 96-well microtiter plates, doubling dilution in phosphate buffer, 0.5% RBCs (*v*/*v*), and 4 hemagglutinating units (HAU) of AIV-H9N2 antigen. Positive groups had at least one serum sample with titer >4.

**Histopathological examination**

Immediately following euthanasia at 35 days old, chicken trachea, lungs, liver, kidney, bursa and thymus specimens were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, blocked in paraffin and cut into 5-µm-thick sections using a rotary microtome (Bancroft and Gamble 2002). The obtained tissue sections were stained with hematoxylin and eosin (H&E) for light microscopy examination.

**Detection of viral shedding**

Viral shedding was detected using 10 tracheal swabs collected at 3 and 5 dpi and inoculated in SPF chicken eggs (9 days old) via allantoic sac route (Reed and Muench 1938). Slide hemagglutination (HA) test was used to confirm the positivity of the allantois fluid and the mean viral titer of pooled allantoic fluid was calculated.

**Statistical analyses**

Statistical analysis of raw data was performed using SAS® software by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) to detect differences between the groups. The experimental data are expressed as the mean ± SEM, and significant differences among the groups were set at a value of $p \leq 0.05$.

## RESULTS

**Clinical observations, mortalities and body weight**

Clinically, H9N2–inoculated chickens (Group IV) showed clinical signs of depression, anorexia, respiratory manifestation as facial edema (sinusitis), sneezing and mild conjunctivitis. In addition, tracheal exudates and air saculitis were more pronounced. Compared with control, AFs-intoxicated chickens displayed a marked decrease in feed consumption with stunted growth. Internally, AFs–intoxicated broilers showed marked enlargement of the liver with abnormal discoloration and ureters distended with uric acid. There were no mortalities in the control and H9N2–inoculated chicks, although there were 11% in AFs–treated chickens (Group II) and 20% in AFs–treated H9N2–inoculated chickens (Group III). The average body weight of broiler chicks was recorded on a weekly basis until the end of the experiment. Initially, there were no significant differences in the body weight among different groups at day 1 of the experiment. However, from day 7, aflatoxicated broilers challenged with H9N2 virus (Group III),

then AFs–treated chicks (Group II) and from day 28, H9N2–inoculated birds (Group IV) exhibited a significant ($p \leq 0.05$) reduction in body weight compared with controls.

**Hepatic enzymes and cell-mediated immunity**

*Liver function*: Compared with the controls, the activities of serum AST and ALT enzymes were significantly ($p \leq 0.05$) enhanced in aflatoxicated broilers (Groups II, III) (Table. 1).

*Total leukocyte count (TLC)*: At 27 days (4 dpi), TLC was significantly lowered ($p \leq 0.05$) in AFs–intoxicated H9N2–challenged ($687.2 \times 10^3/\mu l$), and AFs–treated broilers ($708.8 \times 10^3/\mu l$) compared with control values ($1005.3 \times 10^3/\mu l$). At 33 days (10 dpi), it was still significantly ($p \leq 0.05$) lowered in AFs–intoxicated ($949.2 \times 10^3/\mu l$), and AFs-intoxicated H9N2–challenged broilers ($1407.7 \times 10^3/\mu l$) compared with control values ($1717.9 \times 10^3/\mu l$). However, it was significantly ($p \leq 0.05$) increased in H9N2–challenged chickens ($1270.8 \times 10^3/\mu l$) compared to control birds and AFs–intoxicated birds at 4 dpi, which was returned to control at 10 dpi (Table 2).

*Differential leukocyte count (DLC)*: Leukocytic cells were differentiated into lymphocytes, heterophils, monocytes and eosinophils as percentages of TLC. Table 2 shown that DLC reflected significant ($p \leq 0.05$) increase in the percentage of lymphocyte and heterophile (lymphocytosis and heterophilia) in experimental AFs and/or H9N2–exposed chickens (Groups II-IV) at 4 dpi compared with control. H9N2–challenged chickens (Groups III, IV) showed significant ($p \leq 0.05$) increment in eosinophilic % (eosinophilia). As well, monocytes % were significantly ($p \leq 0.05$) increased in AFs-intoxicated H9N2–challenged chickens only as compared with other experimental groups. At 33 days (10 dpi), lymphocyte was significantly lowered ($p \leq 0.05$) by 69.6% in AFs–intoxicated chicks (Group II), while there was a significant ($p \leq 0.05$) increment in heterophile by 27.2% compared with other experimental groups. H9N2–challenged chickens (Group IV) showed significantly higher ($p \leq 0.05$) eosinophil (1.8%) compared with other groups. There were no significant changes observed in monocytes.

*Lymphocytes transformation activity*: Compared to control, lymphocytes transformation activity was significantly ($p \leq 0.05$) lowered in AFs–treated chickens (Groups II, III) throughout the experiment, with no significant change in H9N2–inoculated chickens at 14 and 27 days (4 dpi) old. Although, it had been significantly increased ($p \leq 0.05$) in all chicken groups at 33 days old (10 dpi), particularly in H9N2–challenged chickens (Group IV) (Table 3).

*Macrophages phagocytic activity*: At 14 days old, aflatoxicated chickens (Groups II, III) expressed significantly ($p \leq 0.05$) higher macrophage phagocytic activity compared to control and H9N2–challenged chickens. However, at 27 and 33 days old (4 and 10 dpi, respectively), they had significantly lower ($p \leq 0.05$) macrophage phagocytic activity, with the lowest activity in AFs+H9N2–challenged chickens at 4 dpi, and AFs–intoxicated chickens at 10 dpi compared with the control. Generally, there is no significant changes had been observed in macrophage phagocytic activity between H9N2-challenged chickens and control (Table 3).

**Humoral-mediated immunity (HI)**

At day 1, there was no significant difference in maternal antibodies titer among all chicken groups. At 33 days (10 dpi), it was noticed that chickens non-inoculated with H9N2 virus (Control and AFs–intoxicated chickens) had no HI antibodies. Meanwhile, H9N2–challenged chickens expressed HI antibody titer, that was significantly lower ($p \leq 0.05$) in AFs+H9N2 than H9N2-challenged chickens (5.5 and 6.3 Log2, respectively) (Table 3).

**Histopathologic assessment (Table 4)**

*Liver*: The severity of hepatic lesion varied between treated chicken groups that were apparently normal livers in the control group (Fig. 1Aa), mildest in H9N2–inoculated chickens (Group IV). More severe lesions appeared in AFs–intoxicated chickens (Group II), where severe granulocytes infiltration within atrophied hepatocytes were seen (Fig. 1Ab). However, the most severe hepatic lesions; severe granulocytes infiltration with destructed hepatocytes, severe ballooning degeneration of hepatocytes with fatty changes and hyperplasia of stellate cells were seen in AFs+H9N2 group (Fig. 1Ac, d).

*Kidneys*: Kidneys of control birds revealed normal histological appearance (Fig. 1Ba). However, both AFs (Group II, Fig. 1Bb) and H9N2 (Group IV, Fig. 1Bc) exerted a harmful effect on kidneys; tubular cast, pronounced intertubular congested blood vessels with degenerated tubular epithelium. This effect was exaggerated by their combination, which exhibits focal intertubular-histeocyte aggregations (Fig. 1Bd).

**Table 1.** Effect of aflatoxins (200 ppb) and/or infection with AIV-H9N2 ($10^6$ $EID_{50}$/ml) on the morality incidence, weekly average body weight (g) and serum liver function biomarkers of broiler chickens

| Groups | Mortality (%)[a] (*n*= 45) | Average body weight (g) (*n*= 45) | | | | | | Liver enzymes (*n*= 20) | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 day | 7 days | 14 days | 21 days | 28 days | 35 days | AST (IU/ml) | ALT (IU/ml) |
| CTR | 0 | 39.2±0.58[a] | 164.0±1.18[a] | 290±7.41[a] | 876.4±22.40[a] | 1532±20.83[a] | 1850±35.35[a] | 35.0±1.14[b] | 25.2±1.15[b] |
| AFs | 11 (5/45) | 40.0±0.70[a] | 120.4±2.13[b] | 148±24.72[b] | 324.0±72.42[b] | 482±34.26[c] | 560±36.74[c] | 41.4±1.88[a] | 29.2±1.50[a] |
| AFs+H9N2 | 20 (9/45) | 40.8±0.58[a] | 123.0±1.41[b] | 158±18.54[b] | 302.0±52.66[b] | 500±41.83[c] | 530±25.49[c] | 41.0±2.30[a] | 29.8±1.20[a] |
| H9N2 | 0 | 40.2±0.86[a] | 162.2±0.81a | 298±6.63[a] | 879.0±21.93[a] | 1020±25.49[b] | 1240±43.01[b] | 35.4±1.12[b] | 24.8±1.43[b] |

Values have different superscripts within the same columns are significantly different at $p \leq 0.05$.
[a] At the end of the experimental period.

**Table 2.** Effect of aflatoxins (200 ppb) and/or infection with AIV-H9N2 ($10^6$ $EID_{50}$/ml) on the total and differential leucocytes of broiler chickens

| Groups | Total leucocytes count ($10^3$/µl) | Differential leucocytes percent | | | |
|---|---|---|---|---|---|
| | | Lymphocytes (%) | Heterophils (%) | Monocytes (%) | Eosinophils (%) |
| ***4 dpi*** | | | | | |
| **CTR** | 1005.3±31.8[b] | 62.2±2.75[a] | 36.2±2.85[a] | 1.6±0.24[b] | 0.0±0.00[c] |
| **AFs** | 708.8±27.1[c] | 75.8±0.58[b] | 22.6±0.60[b] | 1.6±0.24[b] | 0.0±0.00[c] |
| **AFs+H9N2** | 687.2±7.8[c] | 77.5±1.04[b] | 19.3±1.25[b] | 2.3±0.25[a] | 1.0±0.00[b] |
| **H9N2** | 1270.8±81.0[a] | 74.6±0.67[b] | 22.4±0.81[b] | 1.4±0.24[b] | 1.8±0.37[a] |
| 10 dpi | | | | | |
| **CTR** | 1717.9±61.9[a] | 72.4±1.65[b] | 24.8±0.20[b] | 1.8±0.21[a] | 1.0±0.00[a] |
| **AFs** | 949.2±29.17[c] | 69.6±1.56[a] | 27.2±1.39[a] | 2.0±0.00[a] | 1.2±0.22[a] |
| **AFs+H9N2** | 1407.7±66.78[b] | 73.2±0.37[b] | 23.0±0.31[b] | 2.0±0.01[a] | 1.8±0.21[b] |
| **H9N2** | 1560.5±62.4[ab] | 73.5±0.64[b] | 23.0±0.40[b] | 2.0±0.03[a] | 1.5±0.20[a] |

Values have different superscripts within the same column are significantly different at $p \leq 0.05$ ($n = 20$).

**Table 3.** Effect of aflatoxins (200 ppb) and/or infection with AIV-H9N2 ($10^6$ $EID_{50}$/ml) on the lymphocytes transformation and macrophages phagocytic activities, and HI antibody titer (Log2) of broiler chickens

| Groups | Lymphocytes transformation activity (optical density) | | | Macrophages phagocytic activity (µM/ml) | | | Log2 HI GMT titers | |
|---|---|---|---|---|---|---|---|---|
| | 14 days | 27 days (4 dpi) | 33 days (10 dpi) | 14 days | 27 days (4 dpi) | 33 days (10 dpi) | Maternally-derived antibodies | 33 days (10 dpi) |
| **CTR** | 2.35±0.06[a] | 1.42±0.03[a] | 1.86±0.06[a] | 12.6±0.80[a] | 12.42±0.40[a] | 12.10±0.22[a] | 5[a] | 0[a] |
| **AFs** | 1.59±0.08[b] | 1.11±0.05[b] | 1.39±0.05[b] | 29.1±0.49[b] | 9.90±0.27[b] | 6.05±0.13[b] | 5[a] | 0[a] |
| **AFs+H9N2** | 1.53±0.05[b] | 0.97±0.03[b] | 3.32±0.01[d] | 29.1±0.46[b] | 6.12±0.20[c] | 8.77±0.12[c] | 5[a] | 5.5±0.34[b] |
| **H9N2** | 2.34±0.05[a] | 1.41±0.06[a] | 4.33±0.08[c] | 12.5±0.84[a] | 10.33±0.22[a] | 12.99±0.29[a] | 5[a] | 6.3±0.26[c] |

Mean values have different superscript letter within the same column are significantly different between experimental groups at $p \leq 0.05$ ($n = 20$).

**Table 4. The severity of histopathological lesions in experimental chickens**

| Groups | Liver | Kidneys | Lungs | Trachea | Bursa of Fabricius | Thymus |
|---|---|---|---|---|---|---|
| **CTR** | 0 | 0 | 0 | 0 | 0 | 0 |
| **AFs** | 3 | 3 | 3 | 0 | 2 | 1 |
| **AFs+H9N2** | 4 | 3 | 4 | 1 | 3 | 2 |
| **H9N2** | 2 | 2 | 2 | 1 | 2 | 1 |

(0) no lesions; (1) mild lesions; (2) moderate lesions; (3) severe lesions and (4) very severe lesions

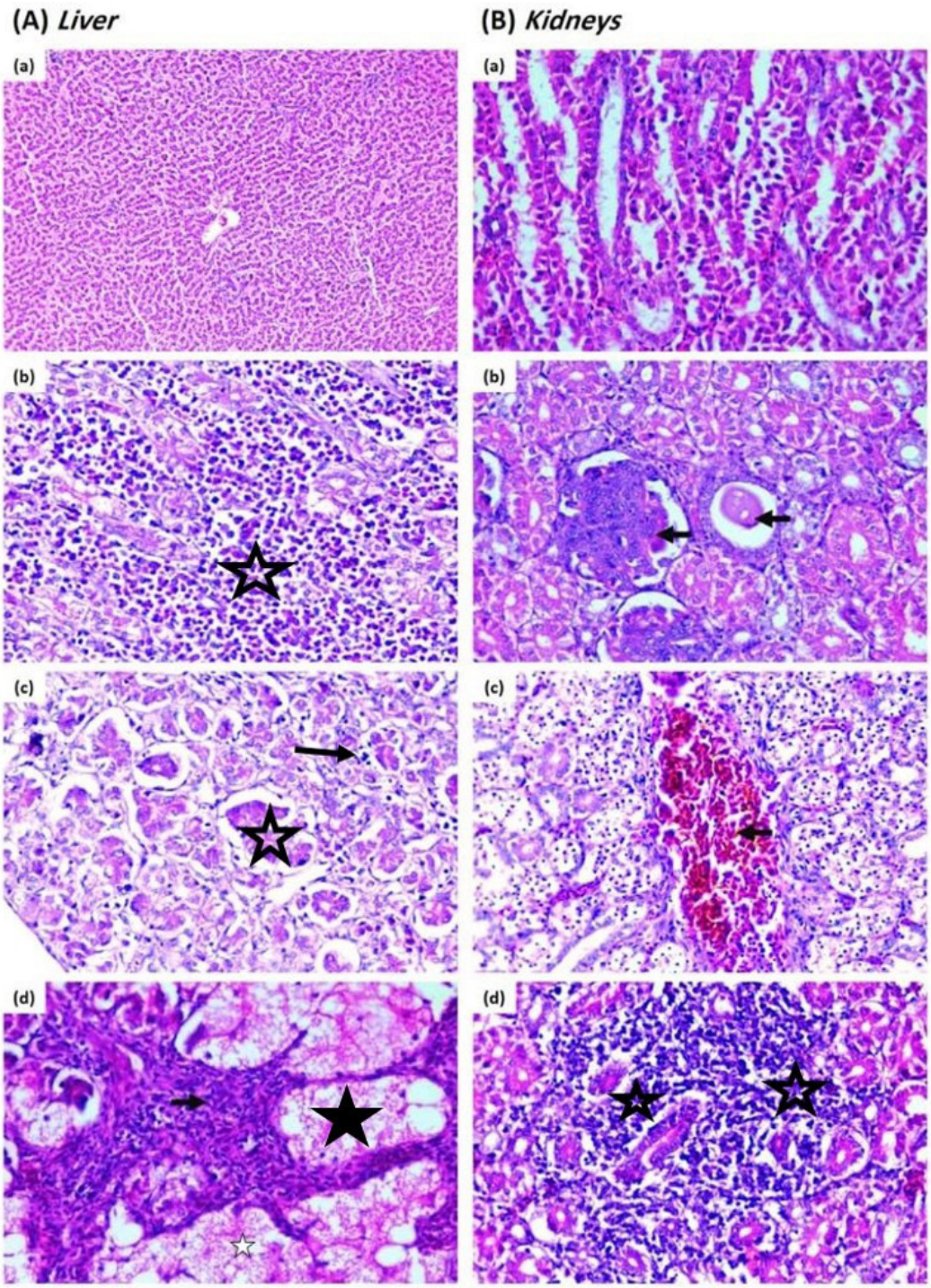


**Figure 1:** Representative photomicrographs of H&E-stained liver and kidneys tissues following aflatoxins and/or H9N2-AIV challenge.

Light microscopy of hepatic tissues from control (a) shows the normal histologic structure of liver tissues. AFs (200 mg/kg ration)–treated broiler chicks (b) exhibited granulocytes infiltration within atrophied hepatocytes (×400). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks showed granulocytes infiltration with destructed hepatocytes, ballooning (c) and degeneration of hepatocytes with fatty changes (arrow) and hyperplasia of stellate cells (stars) (d, H&E ×400).

Light microscopy of renal tissues from control (a) shows the normal histologic structure of renal tissues (×100). AFs (200 mg/kg ration)–treated broiler chicks (b) exhibited showed tubular cast (arrows, ×400). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (c) showed pronounced intertubular congested blood vessels with degenerated tubular epithelium (arrow, ×400). H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (d) showed focal intertubular histocytes aggregations (×400).

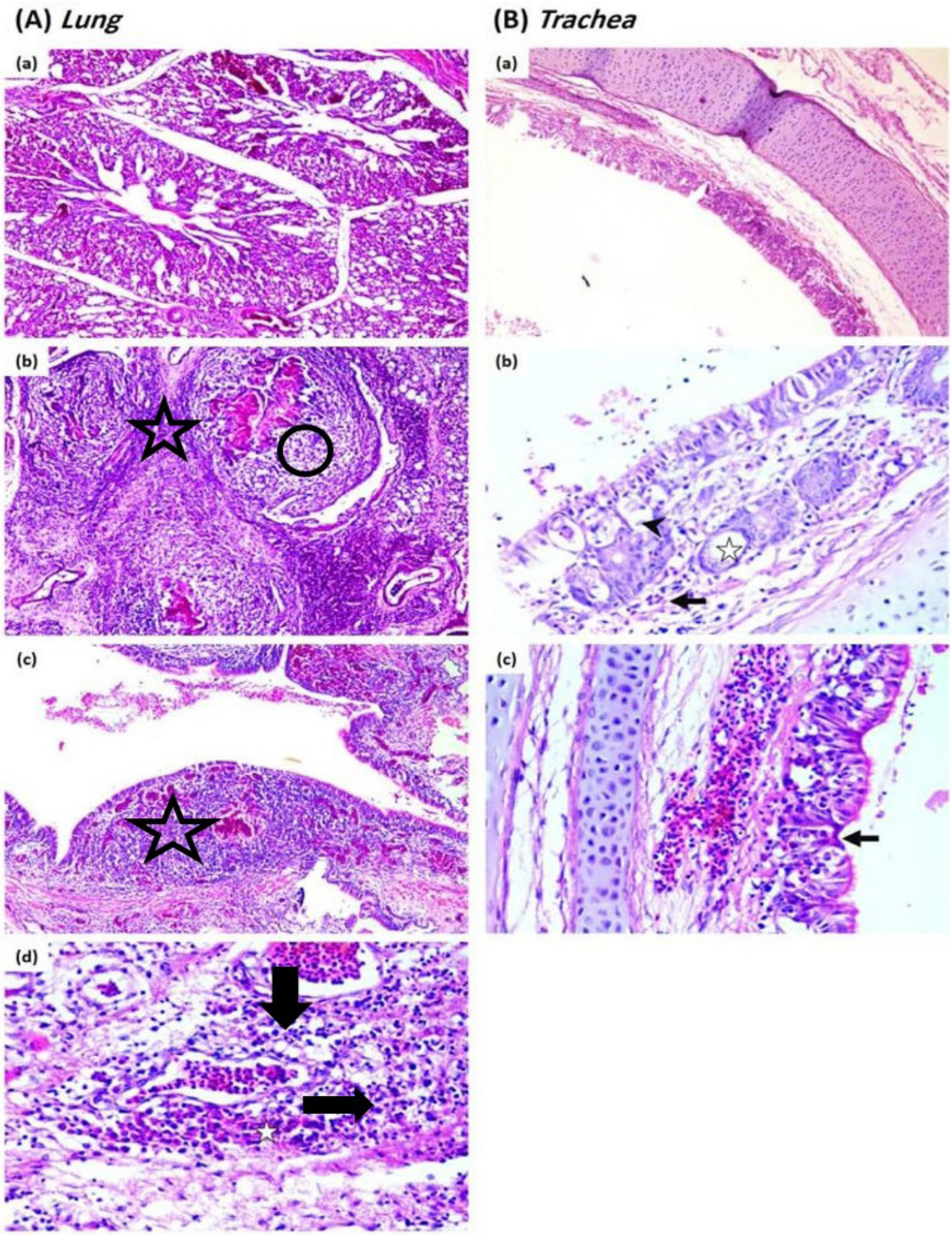


**Figure 2:** Representative photomicrographs of H&E-stained lung and tracheal tissues following aflatoxins and/or H9N2-AIV challenge.

Light microscopy of pulmonary tissues from control (a) shows apparently normal histologic structure (×100). AFs (200 mg/kg ration)–treated broiler chicks (b) showed bronchioles surrounded by heterophils, macrophages, epithelioid cells (star) and multinucleated giant cells and connective tissue (arrow) (×400). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (c) showed thickening of bronchial lining due to mononuclear cells infiltration (star), congestion and hemorrhage (×100). H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (d) showed interstitial granulocytes and mononuclear cells infiltration (star) (×400).

Light microscopy of tracheal tissues from control (a) and AFs (200 mg/kg ration)–treated broiler chicks show apparently normal histologic structure (×100). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (b) showed vacuolation of lining epithelium (star), increased mucous glands (arrowhead) with inflammatory cell infiltration (arrow) (×400). H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (c) showed focal deciliation with submucosal hemorrhage (arrow) (×400).

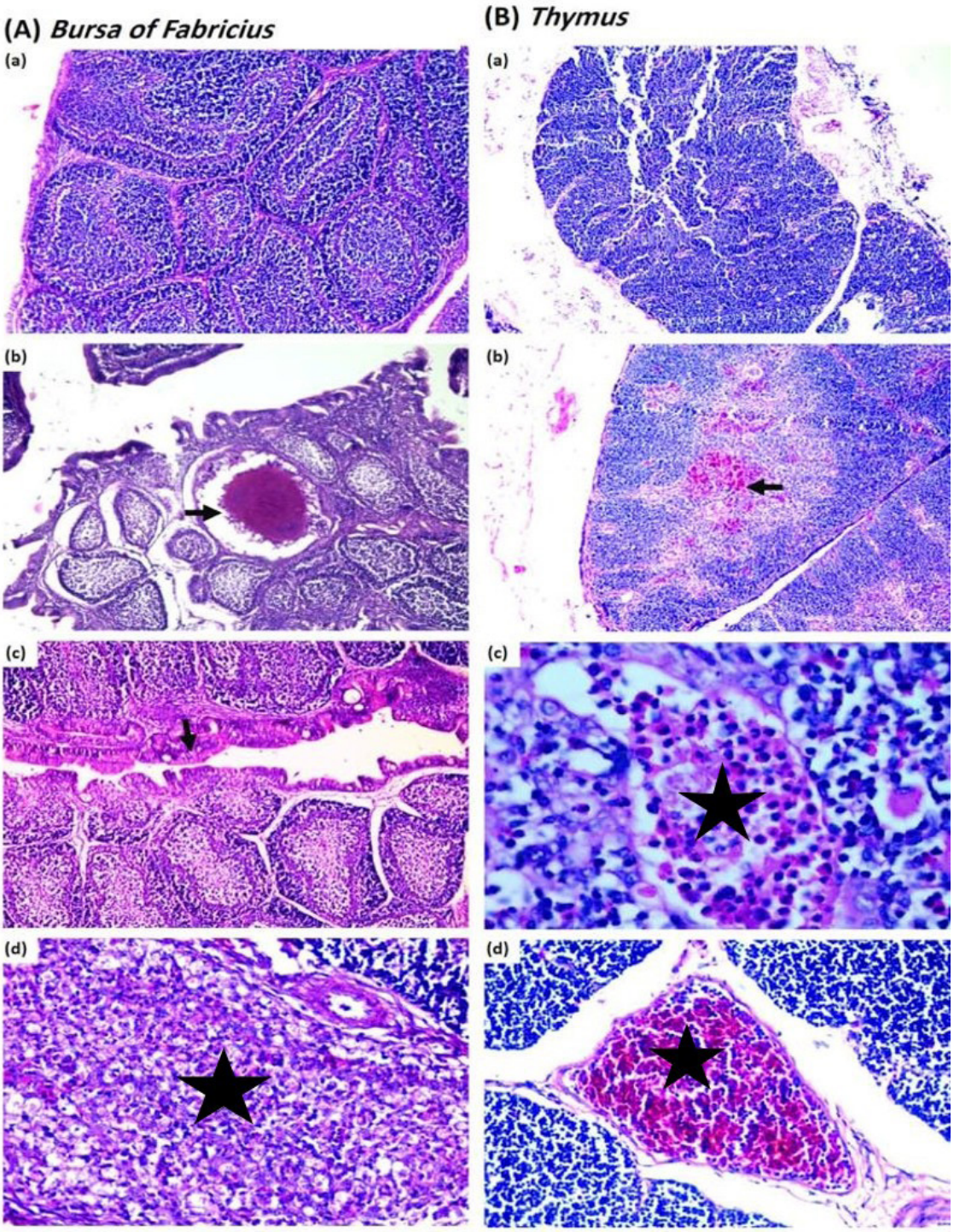


**Figure 3:** Representative photomicrographs of H&E-stained bursa of Fabricius and thymus tissues following aflatoxins and/or H9N2-AIV challenge.

Light microscopy of bursa of Fabricius tissues from control (a) shows apparently normal histologic structure (×100). AFs (200 mg/kg ration)–treated broiler chicks (b) showed granuloma (arrow) within bursal follicle cyst (×100). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (c) showed hyperplasia of lining epithelium with depletion of lymphocytes (arrow) (×100). H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (d) showed interstitial granulocytes and mononuclear cells infiltration (star) (×400).

Light microscopy of thymus tissues from control (a) shows apparently normal histologic structure (×100). AFs (200 mg/kg ration)–treated broiler chicks (b) showed focal hemorrhage in the medulla (arrow) (×100). AFs (200 mg/kg ration) + H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (c) showed widening of Hussal's corpuscles forming cysts containing necrotic debris and granulocytes (star) (×400). H9N2 (100 µl of $10^6$ $EID_{50}$/bird)–challenged broiler chicks (d) showed congested blood vessels (star) (×100).

*Lungs*: Pulmonary tissues from control show apparently normal histologic structure (Fig. 2Aa), while AFs–treated broiler chicks showed bronchioles surrounded by heterophils, macrophages, epithelioid cells and multinucleated giant cells and connective tissue (Fig. 2Ab). Severe thickening of bronchial lining appeared due to mononuclear cells infiltration, congestion and hemorrhage were seen in AFs+H9N2-exposed chickens (Fig. 2Ac), while H9N2–inoculated chickens showed a moderate degree of pneumonia exhibited as thickening of bronchial lining due to mononuclear cells infiltration (Fig. 2Ad).

*Trachea*: The tracheal tissues from control (Fig. 2Ba) and AFs-treated broiler chicks show apparently normal histologic structure. They also were mild in other groups; vacuolation of lining epithelium and increased mucous gland with inflammatory cell infiltration in chickens of AFs+H9N2–inoculated chickens (Group III) (Fig. 2Bb) and focal deciliation in H9N2–challenged chickens (Group IV) (Fig. 2Bc).

*Bursa of Fabricius*: The control chickens (Group I) showed apparently normal bursa (Fig. 3Aa). The severity of lesions in bursa was moderate in chickens in AFs-intoxicated chickens (Group II) – granuloma within bursal follicle cyst (Fig. 3Ab), followed by H9N2–inoculated chickens (Group IV), which appeared as hyperplasia of lining epithelium with depletion of lymphocytes (Fig. 3Ac). There was severe necrosis of plical lymphocytes in AFs+H9N2 group (Fig. 3Ad).

*Thymus*: Thymus of control chickens showed normal architecture (Fig. 3Ba). The severity of lesions in the thymus was mild – focal hemorrhage in the medulla in AFs (Fig. 3Bb) and H9N2 (Fig. 3Bd). They are exaggerated to moderate thymic lesions in AFs+H9N2 group (Fig. 3Bc).

### H9N2-avian influenza viral shedding

As shown in Table 5, all examined tracheal swabs from H9N2–inoculated chickens (Groups III, IV) at 3 dpi, were positive for virus isolation in ECE, which confirmed by HA and HI test using H9N2 antiserum. The H9N2-AIV virus was isolated from 5 of 10 tracheal swabs with a mean viral titer of pooled allantois fluid of $10^{5.6}$ $EID_{50}$/ml at 5 dpi, from H9N2–inoculated chickens (Group IV), meanwhile in AFs+H9N2 group, the virus was isolated from 7 of 10 tracheal swabs with a mean viral titer of $10^{7.8}$ $EID_{50}$/ml.

**Table 5.** H9N2 avian influenza virus shedding in tracheal swabs in experimental chickens at 3 and 5 days post-infection (dpi)

| Groups | 3 dpi | 5 dpi | Mean viral Titer at 5 dpi ($EID_{50}$/ml) |
|---|---|---|---|
| **CTR** | 0/20 | 0/20 | - |
| **AFs** | 0/20 | 0/20 | - |
| **AFs+H9N2** | 20/20 | 10/20 | $10^{7.8a}$ |
| **H9N2** | 20/20 | 14/20 | $10^{5.6b}$ |

Values have different scripts at the same row are significantly different at $p \leq 0.05$ ($n = 20$)

## DISCUSSION

The productivity of poultry farms was scarcely affected by feed contaminated with AFs and their subsequent impacts on broiler performance. Aflatoxicosis and AIV are not only lead to huge economic losses in the poultry industry but also cause serious public health threats worldwide. In broiler, AFs in the diet can act as a stress factor to increase the susceptibility to, or severity of, AIV subtype H9N2 (El Miniawy et al. 2011; Umar et al. 2015). Increased susceptibility of AFs-intoxicated broilers to various bacteria and viruses indicates an impaired immune response and a failure of vaccinal immunity (Subler et al. 2006; Umar et al. 2015). In the pathogenesis of AIV subtype H9N2 in broilers intoxicated with AFs, there was a detrimental clinical picture of respiratory distress involving impairment of chicken performance in addition to hemato-biochemical, histopathological and immunological changes.

The current data indicated that AFs and/or AIV subtype H9N2 caused a reduction in body weight (Afzal and Zahid 2004; Gharaibeh 2008; Hablolvarid et al. 2004; Marchioro et al. 2013; Raju and Devegowda 2000; Yunus et al. 2011), thereby leading to losses in performance and productivity. These effects in broilers may be due to the greater effect of AFs in the initial growth phase, particularly during the first 21 days of age, when the negative impact on weight gain is irreversible due to inhibition of protein synthesis, reduced absorption of nutrients and lower rate of pancreatic enzymes release (Azizpour and Moghadam 2015; Oguz et al. 2000; Safamehr 2008). In additions, AFs provoked a nutritional deficiency and hence, inhibition of weight gain, hepatomegaly and increased weights of visceral organs (Kubena et al. 1990). The suppressed appetite in aflatoxicated groups is due to the impaired liver and kidneys metabolic activity subsequently to the impaired hepato-renal structure (Or-

tatatli et al. 2005; Verma et al. 2010), which appeared clearly in the histopathological deterioration and the elevation of liver enzymes (AST and ALT), however, both enzymes didn't show much high significant increase as the low dose of AFs (200 ppb) induced toxicity to the cell-mediated immunity more than liver (Giambrone et al. 1985). The nephrotoxic effect of AFs may be due to interference with transport function in collecting tubule cells together with diffused impairment of the function of the proximal tubule (Ortatatli et al. 2005). Herein, the mortality began after 14 days of age, which could be attributed to the cumulative effect of AFs.

Infection with LPAI subtype H9N2 is usually mild and localized to the respiratory and intestinal tracts due to the restriction of trypsin-like proteases. However, one of the indicators of pathogenicity after H9N2 experimental infection is the daily observed clinical signs with special attention to feed consumption, body weight and FCR (Gharaibeh 2008). Also, H9N2 infection is associated with edema in the head and neck, conjunctivitis as well as tubulointerstitial nephritis, turbidity of the thoracic and abdominal air sacs, mild congestion of the trachea and lung, mild accumulation of fibrinous exudate on the tracheal mucosa and all these lesions should have a direct negative effect on feed consumption and FCR (Hadipour et al. 2011). Also, decrease in feed consumption, depression and diarrhea, hemorrhage in small intestine and pancreas as well as swollen kidneys with no mortalities were reported (Hs et al. 2016; Somayeh Asadzadeh 2011). Moreover, it appeared that the H9N2 was more pathogenic in aflatoxicated–chicken, which caused more severe clinical signs and pathologic changes in the liver, kidneys, lungs and trachea compared with AFs–treated chickens (Hadipour et al. 2011), leading to potent decrease in feed intake as well as body weight. Also, the mortality rate in AFs+H9N2 exposed chickens (Group III) increased by about 50% than AFs-intoxicated chickens (Group II) (>11%), however, this was not highly significant to be reflected on the estimated liver enzymes (AST and ALT), which may be attributed to the delayed H9N2 infection at 23rd day of age in the AFs-intoxicated birds (from first day of age) that might affect the mortality rate more than the liver enzymes at 27 and 33 days of age (only 4 and 10 dpi) as the respiratory tract is the main target tissue during the pathogenesis or acute infection of H9N2. Previous studies found that H9N2 AIV infections could decrease growth rate and FCR in broilers due to co-infection with other pathogens as IBV, *Staphylococcus aureus*, *Avibacterium paragallinarum* and *E. coli* or immune suppression, which exacerbates H9N2 infection in chickens (Kishida et al. 2004).

It is well known that the suppression of immune function has led to an increase in the susceptibility to a variety of bacterial or viral diseases in chickens (Subler et al. 2006). Previously available data on the pathogenesis of LPAI viruses in chickens suggested that the H9N2 LPAI viruses in poultry flocks resulted in diverse clinical syndromes of varying severity depending on the viral strain, as well as the co-infection with immunosuppressive diseases (Bano et al. 2003; Kishida et al. 2004). It was indicated that aflatoxicosis has an immunosuppressive effect in broiler chickens reflected by lower activity of lymphocyte transformation in all stages of the experiment. Hatori et al. (1991) found that the immune responses mediated by T cells appeared to be sensitive to AFs. Even the very low levels of AFs can disturb the immune system of birds, and thus in association with other pathogens (Ellakany et al. 2011) it can exacerbate disease outcomes. Many studies conducted in poultry showed that exposure to AFs–contaminated feed resulted in suppression of the cell-mediated immune responses, thymic aplasia, reduction of the function and number of T-lymphocyte, suppressed phagocytic activity and reduced complement activity. Impairment of cellular function by AFs seems to be due to its effects on the production of lymphokines and antigen processing by macrophages, as well as a decrease in or lack of the heat-stable serum factors involved in phagocytosis (Cusumano et al. 1996; El Miniawy et al. 2014; Raisuddin et al. 1990; Theumer et al. 2010). The immunosuppressive effect of AFs was further clearly noticed by the lower macrophage phagocytic activity and TLC (Abdel-Wahhab et al. 2002; Basmacioglu et al. 2005; Celik et al. 2000; Oguz et al. 2000), particularly after challenge with H9N2. Lymphocytes and WBC were decreased and heterophils were increased without any noticeable influence on monocyte and eosinophil in male broiler chickens exposed to mycotoxins naturally contaminated diets (Mohaghegh et al. 2016). The possibility of phagocytic depression during mycotoxicosis may be due to inhibition of DNA, RNA and protein synthesis in macrophages after mycotoxins exposure; or due to alterations in metabolic processes, principally glycolysis essential for phagocytosis (Qureshi and Hagler 1992). Moreover, it may be attributed to an alteration in the macrophage membrane. These functional alterations may affect RNA function on actin and myosin formation that

are essential for chemotaxis and phagocytosis (Aderem and Underhill 1999). Neldon-Ortiz and Qureshi (1991) pointed out that morphological change in chicken peritoneal macrophages after AFs exposure included a decline in adherence ability, blebbing formation on the cellular surface, and nuclear disintegration. AFs may interfere with the lipopolysaccharide (LPS) binding to protein on CD14 causing down-regulation of its expression decreasing the induction of cytokines (Wright et al. 1990).

H9N2-avian influenza viral shedding results figured out the role of aflatoxicosis in increasing the number of birds shedding the H9N2 virus and increased virus titer ($10^{7.8}$ $EID_{50}$/ml) in the AFs–treated chickens than the only H9N2–inoculated birds ($10^{5.6}$ $EID_{50}$/ml), further indicating the immunosuppressive effect of the aflatoxicosis. In turkeys, Umar et al. (2015) reported that H9N2 virus infection during aflatoxicosis characterized by a higher level in viral shedding in oropharyngeal swabs. In addition, AFs can induce immunosuppression in the form of impaired T or B lymphocytes performance, decreased antibody synthesis, and decreased interferon and macrophages activity (Umar et al. 2012). Generally, aflatoxicosis decreases the resistance to common infectious diseases because of impairment of humoral and cellular immune responses (Bakshi et al. 2000). The immunosuppression induced by aflatoxicosis led to increased H9N2 pathogenicity (Kwon et al. 2008), which in turn can lead to mortality in H9N2–inoculated chickens.

## CONCLUSION

From all the previous data we can conclude that AFs and H9N2 virus had an immunosuppressive effect. It was clearly appeared in their pathological changes exaggerated during their combination in broiler chickens. AFs contaminations promoting the pathogenesis of H9N2 infection to produce more pronounced clinical signs, histopathological lesions, and mortalities.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Case report
Κλινικό περιστατικό

# An uncommon fetal retention case: Ruptured ventral hernia in a sheep

**C. Peker, E. H. Uçar, O. Varoğlu, G. Erdoğan**

*Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, 09100 Işıklı-Aydın, Turkey*

**ABSTRACT.** A four-year-old pluriparous sheep was brought to our clinic with the complaints of mild anorexia and a wound on the ventral abdominal area, with part of a dead lamb protruding from this lesion. Clinical examination revealed that there was a fetal retention into the ruptured gravid uterine horn that was trapped within a ventral hernia. At herniorrhaphy after removing the dead fetus, strong connections between the uterine and abdominal wall and chronic scars/necrosed tissues were detected in the wound edges, which revealed the long time lapse between the unnoticed herniation-tearing and surgery. Although many ventral hernia cases have been reported in ovine pregnancies, the high maternal resilience in this ruptured hysterocele case, which had a two-week-old history at minimum, is clinically remarkable.

***Keywords:*** Ventral hysterocele, uterine rupture, fetal retention, sheep

*Corresponding Author:*
Erdoğan G., Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, 09100 Işıklı-Aydın, Turkey
E-mail address: gunesems@yahoo.com

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## INTRODUCTION

Herniation is a well-described pathology in pregnant animals. Ventral deviation and trapping of the pregnant horn are commonly seen in sheep, especially in older animals during late pregnancy (Jackson, 2004; Tiwari et al., 2004; Parvez et al., 2016). Spontaneous or traumatic injuries can result in hernia and falling of the uterus into the herniated portion (Purohit, 2006). Pregnancy is seen to be a contributory factor, because the abdominal wall weakens during this period (Noakes et al., 2009; Jettennavar et al., 2010).

Ventral uterine hernia is grossly visible by swelling/enlargement at the lateral abdomen (Purohit, 2006), especially on the right side (Al-Sobayil and Ahmed, 2010). The swelling is very prominent; it can be located anywhere from the lateral side of the thoracic cavity to the iliac crest, above the stifle (Abdin-Bey and Ramadan, 2001; Mahdi, 2015). Systemic symptoms are usually absent and incarceration risk is considered as low in ventral hernia cases (Venugopalan, 2000). Jettenavar et al. (2010) reported that ventral hernias are generally ignored by the rural farmer community unless they result in some serious symptoms. However, females with a hernia during late pregnancy are at high risk of dystocia due to blocked myometrial contractility and utero-peritoneal adhesions (Smith and Sherman, 1994; Sobiraj, 1994; Erdogan et al., 2015).

In this case report, the clinical findings of a ruptured ventral hysterocele in a sheep were described that, incidentally, had occurred at least two weeks previously.

## CASE REPORT

A four-year-old pluriparous ewe, weighing approximately 50-55 kg, was brought to the Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology clinic. According to farm records, the new flock was bought approximately two weeks earlier and the current problem of this sheep had not been noticed. They had considered that her only problem was a slight loss of appetite for a few days. However, careful observation revealed a ruptured area with a fetid odor.

During clinical examination, the sheep was conscious, walking painlessly, but had mild dehydration. The inspection of the ventral abdomen showed an 8 cm diameter perforation located at the cranial area of the left half-udder. It was seen that the wound edges had fibrous thickness and necrotic scars. The protruded necrotic tail of the dead fetus carried a fetid odor (Fig. 1). After traction of the necrotic tail part, a twisted joint of the fetal limbs was detected in the entrance of the sac (Fig. 2).

**Figure 1.** Aspherical opening with fetid odor at the abdominal region. Part of fetal tail protruding from the wound (white arrows)

**Figure 2.** Appearance of the ventral abdomen following the tracking of fetal tail and preoperative preparation of the ruptured hernia

As no hematological, biochemical or radiographic analysis could be performed due to the owner's financial position, the removal of the dead fetus and surgical repair of the hernia sac were performed. Using a sedative (0.05 mg/kg xylazine HCl, Rompun® 2%, Bayer) and local anesthetic (200 mg lidocaine HCl, Adokain®, Sanovel), the ruptured area was widened with excisions and the dead fetus was successfully removed, being intact. It was male, weighing 2.5 kg, and without any abnormalities (Fig. 3).

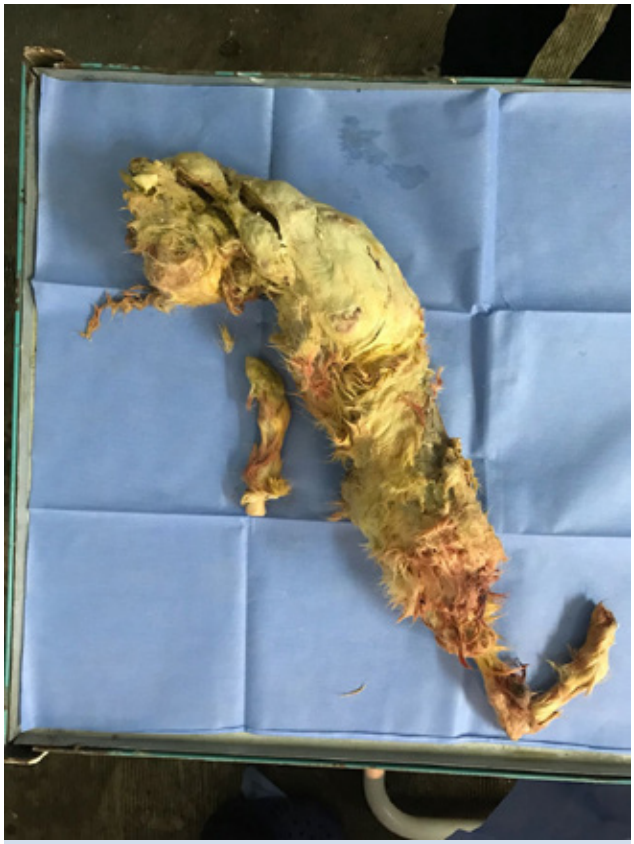

**Figure 3.** Macroscopic appearance of dead fetus

During surgery, the strong adhesions including skin and muscle tissues between the ruptured wall of the gravid horn and ventral abdominal wall were separated bluntly. However, other strong fibrous connections from the gravid horn to the omentum and small intestines were not separated in order to avoid hemorrhage and perforation risk. After intrauterine irrigation with warm saline solution and antibacterial applications (crystallized penicillin G potassium, 2,000,000 IU) to the uterine and abdominal cavities, the uterus was closed and the hernia repaired according to the appropriate repair procedures (Hoise, 2007). All adhesions located along the ruptured area were removed bluntly, and tranexamic acid (10 mg/kg, Transamine®, Fako) was injected to avoid local hemorrhages. Balanced electrolyte solutions (40 ml/kg) and dextrose 5% (20 ml/kg) were provided to the patient animal intravenously. The sheep recovered after a week with the administration of tetracycline HCl (20 mg/kg, Tetraoxyphen L.A.® 20%, Atafen) and flunixin meglumine (1.1 mg/kg, Fulimed®, Alke) injections, post-operatively.

## DISCUSSION

Obstetric emergencies in small ruminants have a negative impact on fetal survival rate and maternal fertility. Among these emergencies, the intrusion of the gravid uterus in the hernial sac is commonly encountered in sheep (Jackson, 2004). Researchers have reported that the most common type of hernia is the ventral abdominal, and its prevalence is reported as between 58.34 and 68.2% of all types of hernias (Al-Sobayil and Ahmed, 2007; Hassen et al., 2017; Mahdi, 2015). The evisceration of the fetus accompanying a ventral abdominal hernia is a very rare condition, especially on the left side, in contrast to Al-Sobayil's and Ahmed's (2010) findings. Only one similar case report complicated by fetal lamb evisceration was found in the accessed veterinary literature, but it could not be treated surgically due to a large hernia sac and lesions on the skin. Perez et al. (2002) emphasized the possible deleterious impacts of laparoscopic insemination procedures on the abdominal wall.

Extreme abdominal distention, weakness of the abdominal muscles, and different types of mechanical traumas (kick, horn thrust, and blunt objects) are important contributory factors for hernia formation (Smith and Sherman, 1994; Krishnamurty, 1995; Arthur, 1989; Al-Sobayil and Ahmed, 2007; Hassen et al., 2017). These researchers have pointed to blunt trauma causing muscle and visceral disruptions / contusions without making any external wound. However, the perforation of the hernia sac might be resulted by a second external trauma or fetal movements at parturition complicated with cervical spasm and or stenosis in our case. It is estimated that this situation is more than two-week-old according to owner's explanation and appearance of the edges of the rupture wound and internal adhesions.

Although evaluating of fetal and maternal health condition have not been based on the reliable data of preoperative hematological analysis and patient's history, it can be hypothesized that there are two plausible explanations to evaluate the maternal preoperative "good" condition and successful treatment.

Firstly, according to the weight (about 2.5 kg) and intact appearance of fetus without any abnormality (Fig 3), it can be considered that uterine inertia resulted in the dystocia then, fetal retention. The main cause and time of fetal death are not detected based on clinical findings. It might be after trauma immediately causing evisceration or hypoxia at prolonged second phase of parturition. Maternal resistance may hide the real condition and cause the quiescent days clinically.

In our previously case report, an intact co-twin fetus without causing any systemic disorder was removed from a ventral hernia after 30 days of sibling's birth (Erdoğan et al., 2015). Even though limited reports, the fact that some unknown factors affecting on the maternal response following fetal loss should be considered.

The second plausible explanation is that these strong connective tissues observed in the hernia sac and wound edges might act as a strong barrier to block the peritoneal contamination cause by fetal fluids, especially after the fetal death. It was well-known that the adhesions resulting from hernia, and they are among serosal surfaces due to an imbalance between fibrin deposition and fibrinolysis (Van der Wal and Jeekel, 2007). These adhesions prevent the mobility of uterus within the abdominal cavity resulting in decreased dilatation of cervix and expulsive force at term (Jackson 2004; Purohit, 2006; Erdoğan et al., 2015, Khan et al., 2018). However, this adhesive tissues might be not only the cause of fetal retention, but also had protective effect for maternal toxicities in this case. Additionally, the direction of rupture would make easy drainage of all fetal fluids and inflammatory discharges. The easily drainage of infective fetal fluids and not being contaminant to peritoneum / other abdominal organs and tissues may be an important protective factor for this case. Interestingly, opening the outside of hernia sac helped the fluid drainage and then, the dead fetus obstructed in wound might block the external contamination.

Trauma due to horning from other animals appeared to be the most common cause of abdominal hernias (Al-Sobayil and Ahmed, 2007). Unpredictable hazardous behaviors such as sudden movements, horning, kicking, and other metal stuffs wire fencing can trigger a lethal emergencies in pregnant animals.

In summary, this manuscript presents a clinical case of 4-year-old sheep with ventral hernia. Gravid uterus with dead fetus is rare case in ovine reproductive medicine. ruptured ventral hysterocele complicated with fetal death repaired surgically in this ewe is extremely rare case. Following the pregnancy diagnosis and fetal counting, dividing the pregnant ewes into different groups is essential for a good management system. Some essential approaches in the herd management system (separation pregnant females, grouping them according to fetal counts, eliminating traumatic issues in flock, close observations of animals etc.) would make timely diagnosis and treatment possible to decrease maternal and fetal mortality.

## CONFLICT OF INTEREST

None declared.

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Case report
Κλινικό περιστατικό

# Complete vaginal stenosis and hematocolpus in two bitches with a history of GnRH treatment to postpone puberty

**G. Marino, M. Quartuccio, A. Sfacteria, S. Cristarella, A. Zanghì**

*Department of Veterinary Sciences, University of Messina, Messina, Italy*

**ABSTRACT.** Two mixed-breed bitches (18 and 19 months), that had been treated, one year before, with deslorelin acetate implant to postpone puberty, were hospitalized and monitored during their first heat. The heat was presumed by the owners, that observed vulvar swelling in both cases but no vulvar bloody discharge. The following diagnostic procedures were employed: physical genital tract examination, vaginoscopy, vaginal cytology, endocrine assay, ultrasound and X-ray using vaginal infusion of iodum and pneumobladder as positive and negative contrasts. In bitch 1, vaginal cytology and progesterone levels confirmed the presence of an ovulatory "dry" oestrus, without cytological presence of red blood cells, progressing to dioestrus. Ultrasound showed preovulatory follicles and, in the following days, transition to corpora lutea. The caudal abdomen presented a large ovoid cystic structure filled with echoic fluid, next to the bladder. Radiographic scans demonstrated a normal bladder profile, while the contrast medium failed to enter into the cranial vagina. On the basis of these findings, the bitch 1 was submitted to laparotomy 10 days after the end of oestrus. A vaginal dilatation (10x5 cm), from which brown fluid was aspirated, was found and resected together with uterus and ovaries. Bitch 2 had the same diagnostic route and findings, but she was laparotomized 3 months after the heat. During this period no spontaneous regression of the lesion was observed. At laparotomy, the vaginal dilatation (8x4 cm) was only aspirated and the bitch regularly neutered. In both cases, cytology of the fluid taken from the vaginal sac revealed superficial epithelial cells and abundant degenerate red blood cells. Histology (bitch 1) confirmed the vaginal origin of the dilatation and revealed an additional Gärtner duct cyst. The abnormality (hematocolpus) probably originated by an inadequate drainage of proestrous bloody discharge because of a severe vaginal stenosis. A congenital origin of the lesion was unlikely; it was strongly suspected that the treatment of the prepubertal bitches interfered, by an irreversible way, with the normal development of the vagina.

***Keywords:*** Dog, hematocolpus, deslorelin, ultrasound, pathology

*Corresponding Author:*
Marino Gabriele, Department of Veterinary Sciences - University of Messina
Via Palatucci, Polo Universitario Annunziata, 98168 Messina, Italy
Email address: marinog@unime.it

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## CASE HISTORY

Two mixed-breed female dogs (bitch 1 and 2), not related to each other, treated at 6 months of age with 4.7 mg deslorelin acetate implant (Suprelorin®, Virbac), inserted into the interscapular region, were hospitalized and monitored during their first heat, observed at the age of 18 and 19 months, respectively.

All procedures were approved for ethical implications by the Center for Animal Reproduction and Assisted Insemination of the University of Messina. The extra-label use of the drug, not designed for prepubertal female dogs, was approved by owners in agreement with literature (Trigg et al., 2006; Sirivaidyapong et al., 2012; Marino et al., 2014; Kaya et al., 2015). Even the diagnostic trials had the owners' consent.

At presentation, bitch 1 (Europe) did not show any sign of disorders. The vulva was well developed, increased in volume, dry with wrinkled skin. Digital stimulation of the perivulvar skin evoked a lateral displacement of the tail, accompanied by a stiffening of the posterior limbs. No bloody discharge was observed. Abdominal palpation allowed appreciating an ovoid and firm structure consistent with an enlarged bladder for position and size. The cervix and the uterus were not detectable by palpation. Digital vaginal exploration showed a normally shaped vestibulo-vaginal channel, although slightly stenotic at the cingulus level. Vaginal cytology that was performed at two-day interval during proestrus-oestrus and weekly during dioestrus confirmed a normal transition from proestrus to dioestrus. Cornification during oestrus reached percentage of 80 % lasting approximately 9 days. Neutrophils were detected only at the dioestrus onset, in concomitance to the presence of intermediate and parabasal cells. Red blood cells were not detected in any phase. Vaginoscopy was performed with a rigid endoscope (TCI-Endoscope, length 43 cm, Karl Storz) without sedation, at two-day interval during proestrus-oestrus until dioestrus onset. The introduction of the instrument was facilitated by a shunt system (Minitube), which also provided fixation to the vaginal wall allowing pumping air to facilitate the visualization of the vagina. Typical mucosal folds changed in appearance during the period of study, passing from oedematous, pink, and round (late proestrus) to pale and crenulated (oestrus) up to flattened and flaccid (dioestrus). No septa were observed at every level. The lumen was slightly stenotic and the bottom of the vagina and the cervix were not visualized, giving the appearance of a blinded vagina. Blood sampling was performed every two days until dioestrus onset. The obtained serum was processed for the determination of progesterone, using an Enzyme Linked Fluorescent Assay (BioMeuriex, Minividas). Circulating progesterone progressively increased from values of 0.9 ng/ml at presentation to 24 ng/ml at dioestrus onset. Ultrasound examination of the abdomen (Esaote, MyLab 40 Vet, 8 MHz convex probe, 12 MHz linear probe) was performed every two days until dioestrus onset. At presentation, multiple cavitary structures in the ovaries, with an anechoic content, 0.6-0.8 cm in diameter, compatible with preovulatory follicles were seen. At ovulation, an increase in echogenicity of such structures was evident together with the finding of a small amount of liquid withheld in the ovarian bursa. The uterus showed normal appearance, a diameter of about 1.5 cm and the absence of intrauterine fluid. For the whole period of monitoring, a large cystic oval formation, at least 8-10 cm long and 4-5 cm wide, thick-walled, and with the presence of numerous and dense echoes within the lumen, was found (Fig. 1).

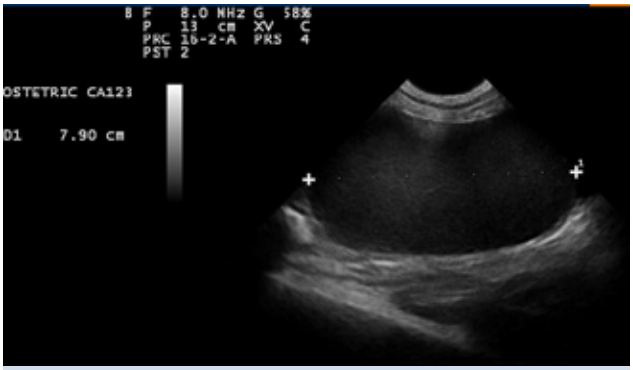


**Figure 1.** Bitch 1 (Europe). Caudal abdomen. Ultrasound appearance of the large oval cystic structure (hematocolpus) with an echoic content.

This undefined cystic formation was found next to a second cystic oval anechoic structure, more attributable to the bladder. The ultrasound-guided catheterisation of the bladder confirmed the nature of the second structure. By contrast, the introduction of ultrasound-guided catheter into the vagina, presented difficulties in the progression, stopping cranially, for the presence of a thin hyperechoic septum (Fig. 2). The abdominal X-ray examination (Univet, 300HS) was carried out on dorsoventral and lateral views. In order to enhance the contrast of the vaginal area, iopamidol (Iopamiro 300, Bracco Imaging) was inoculated in vagina throughout a Foley catheter that was inserted and fixed in the caudal vagina.

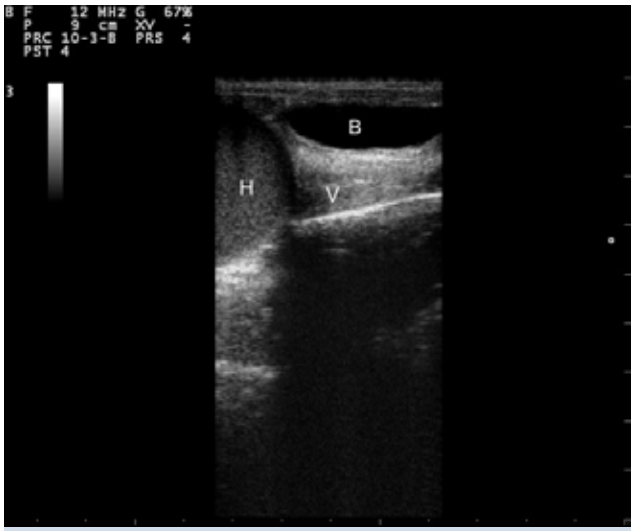


**Figure 2.** Bitch 2 (Moon). Appearance of continuity between the hematocolpus (H) and the vagina (V). Visible even a partial scan of the bladder (B).

The positive contrast medium showed a radiological stop in the vagina, emphasized by the presence of a substantial amount of iodum between the septum and the balloon of the Foley catheter. Then, pneumocystography was obtained by blowing filtered air into the bladder throughout a Foley catheter. The bladder, of normal appearance and content, had no connection with the undefined cystic formation. In light of these findings and the diagnostic suspicion of an abnormal collection in the vagina/uterus, the bitch was submitted to exploratory laparotomy and eventual ovariohysterectomy 10 days after the end of the oestrus. After premedication with intramuscular injections of tramadol hydrochloride (2 mg/kg b.w.; Contramal, Grunenthal Italia) and acepromazine maleate (0.05 mg/kg b.w.; Prequilan, Fatro), the patient was induced with diazepam (0.5 mg/kg b.w.; Diazepam, Intervet) and propofol (3 mg/kg b.w.; Propovet, Zoetis) administered intravenously. After intubation, anaesthesia was maintained with isoflurane in 100% oxygen. Laparotomy was performed on the linea alba. In proximity to the bladder, but in continuity with the uterus cranially and with the vagina caudally, an impressive fluid-filled dilatation was found (Fig. 3), including the cervix. About 200 ml of brown fluid was aspirated from the dilatation (Fig. 4). Furthermore, a second cyst inside the main cystic structure was found, from which 10 ml of yellowish mucoid fluid was aspirated (Fig. 5). The fluids were cytologically evaluated during laparotomy. The first fluid showed only epithelial vaginal cells, many of which were cornified and abundant degenerate red blood cells (Fig. 6).

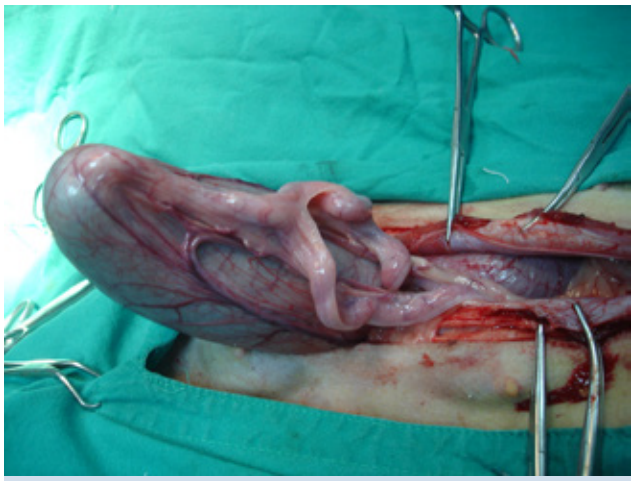

**Figure 3.** Europe. Externalization of the formation caudal to the body of the uterus.

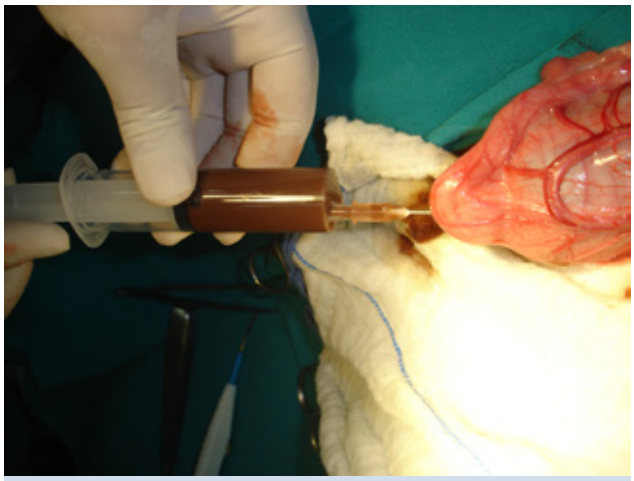

**Figure 4.** Moon. Intraoperative aspiration of the brown fluid contained in the hematocolpus.

**Figure 5.** Moon. Different aspect of the content of the second cavity (Gärtner duct cyst).

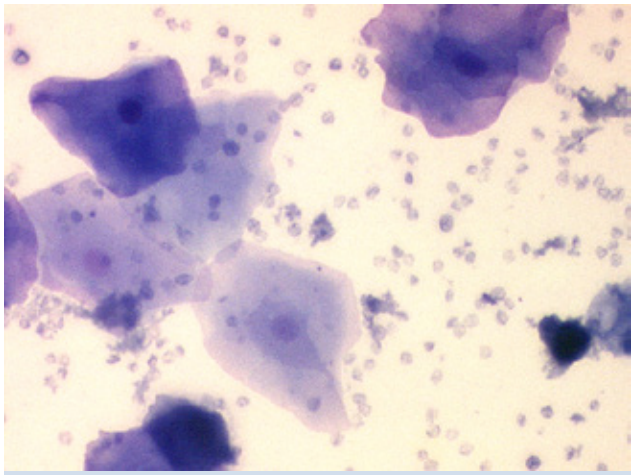

**Figure 6.** Moon. Superficial epithelial cells and degenerate red blood cells in the fluid aspirated from the hematocolpus. May - Grünwald Giemsa. Obv 20x.

The second fluid presented parabasal-like epithelial cells and mucus. In both fluids no bacteria and inflammatory cells were detected. A diagnosis of hematocolpus was done. After ligating the vaginal arteries and branches of the internal pudendal arteries, the dilated vagina was amputated at 2/3 level and removed together with uterus and ovaries. The caudal wall of the dilated vagina was inspected to confirm the absence of communication with the caudal portion of the vagina (Fig. 7).

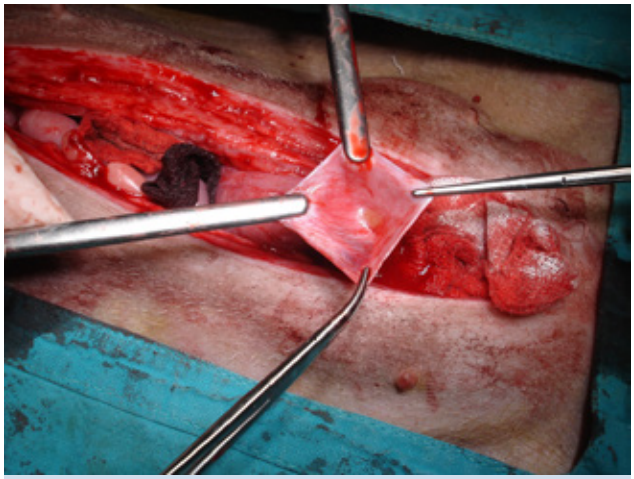

**Figure 7.** Europe. Visualization of the blinded caudal wall of the hematocolpus.

The removed uterine horns were 10 cm length and 1.5 cm in diameter, with a spiral trend according to the phase of the cycle (dioestrus). The ovaries, respectively of 1.5 x 1.0 x 0.5 cm on the right and 2.5 x 1.0 x 0.6 cm on the left, presented both newly formed corpora lutea. The cervix was found inside the dilated area, projecting in it, surrounded by large haemorrhagic areas (Fig. 8).

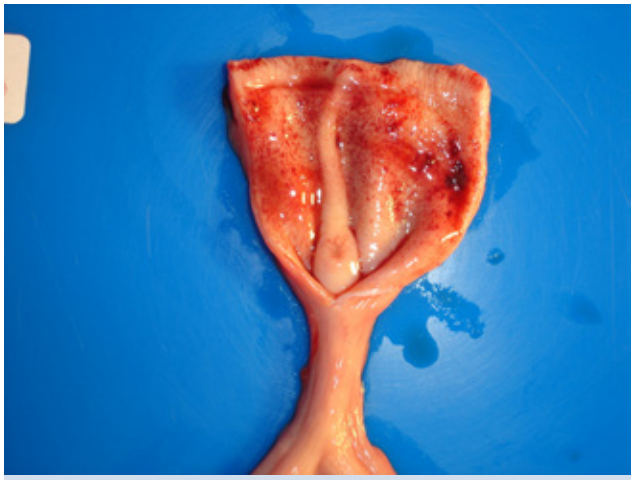

**Figure 8.** Europe. Gross appearance of the mucosa of the hematocolpus with haemorrhagic areas. Following the dorsal median fold the cervix is visualized.

Specimens were processed for histopathological examination. The vaginal mucosa was thinned and partially degenerate, sometimes covered by a multilayered epithelium. In the cranial part, the mucosal epithelium appeared monolayered with strong polarization, similar to endocervical epithelium. Inflammatory cells were not detectable in the mucosa and the underlying layers. In some areas the submucosa was characterized by haemorrhagic extravasation (Fig. 9). A large cavitary structure lined by a single layered epithelium was found resembling a Gärtner duct epithelium (Fig. 10).

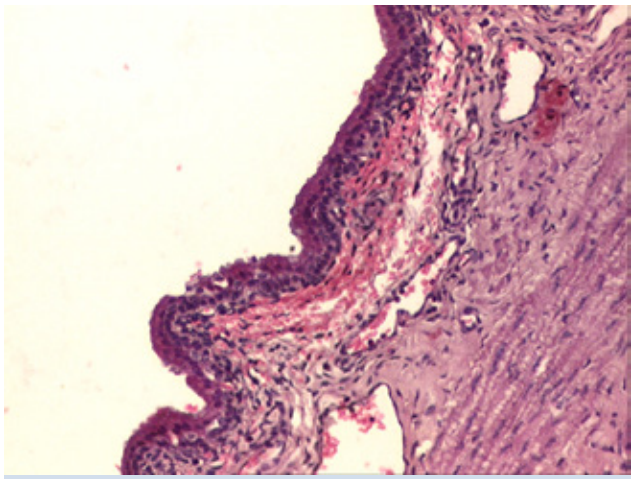

**Figure 9.** Europe. Hematocolpus. Vaginal mucosa close to the cervix covered with a columnar epithelium. Note the absence of inflammatory infiltrates and the hemorrhagic extravasation. Haematoxylin and eosin. Magnification 20x.

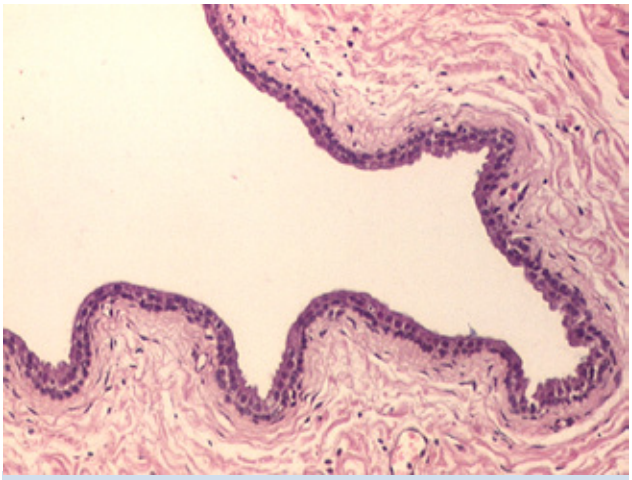

**Figure 10.** Europe. Typical single layered epithelium lining the Gärtner's cyst. Haematoxylin and eosin. Magnification 20x.

Bitch 2 (Moon) had perfectly comparable findings. She was monitored until 3 months after the dry heat. During dioestrus, ultrasound monitoring of the vaginal dilatation was performed weekly, without evident changes in size and appearance. At laparotomy, the main cyst (hematocolpus) and the secondary smaller cyst (Gärtner duct cyst) were only aspirated and left in place; ligature was done at the uterine body level to remove uterus and ovaries. Moon had rectilinear uterine horns of 10.5 cm in length and 0.8 cm in diameter according to the phase of the cycle (anoestrus). The ovaries, sized respectively 1.8 x 0.9 x 0.7 cm on the right and 1.8 x 0.9 x 0.8 cm on the left, presented regressing corpora lutea. The uterine mucosa showed an initial picture of segmental hyperplasia. During follow-up, the stump was monitored by ultrasound and no changes were detected for a period of 1 year after the surgery.

## DISCUSSION

Disorders of the development of the vagina and vestibulum are occasionally reported in the bitch and include vaginal septa, vaginovestibular stenosis, vestibulovulvar stenosis, segmental aplasia of the vagina (Gee et al., 1977; Wadsworth et al., 1978; Holt and Sayle, 1981; Wykes and Soderberg, 1983; Hawe and Loeb, 1984; Root et al., 1995; Archbald and Wolfsdorf, 1996; Kyles et al., 1996). No breed and genetic predisposition for these abnormalities have been reported. Clinical signs reported in dogs with vaginal abnormalities include chronic vaginitis, mating difficulty, urinary incontinence, chronic urinary tract infections, dystocia and ambiguous external genitalia (Holt and Sayle, 1981; Wykes and Soderberg, 1983; Root et al., 1995; Archbald and Wolfsdorf, 1996; Kyles et al., 1996), despite some bitches with vaginal septa or circumferential stenosis are asymptomatic (Wykes and Soderberg, 1983; Root et al., 1995; Kyles et al., 1996). The terms hydrocolpus, mucocolpus, hematocolpus, pyocolpus refer to the abnormal distension of the vagina for the accumulation of fluid, mucus, blood and pus, respectively. These conditions may be related to a difficult drainage of the vagina and the few reported cases in the bitch were generally secondary to developmental disorder of the vestibulo-vaginal tract (Gee et al., 1977; Wadsworth et al., 1978; Hawe and Loeb, 1984; Tsumagari et al., 2001; Viehoff and Sjollema, 2003; McIntyre et al., 2010; Marinho et al., 2013; Alonge et al., 2015).

GnRH agonists are used as anticonceptionals in the canine species. Deslorelin acetate is a GnRH agonist developed as an implant, whose main indication is transitory contraception in adult male dogs. Recent studies have proved the efficacy of the treatment in prepubertal dogs to postpone puberty (Trigg et al., 2006; Sirivaidyapong et al., 2012; Marino et al., 2014; Kaya et al., 2015). There are few data about the side effects of GnRH agonists in veterinary medicine, and they are generally considered safe and totally reversible. Persistent oestrus related or not to ovarian cysts, uterine disorders, urinary incontinence and hair abnormalities have occasionally been described (Arlt et al., 2011; Fontaine and Fontbonne, 2011; Palm and Reichler, 2012). In previous studies on prepubertal bitches, delayed epiphyseal closure, hip dysplasia, transient juvenile vaginitis and marked atrophy of the internal genital tract have been reported with minimal or no clinical impact (Marino et al., 2014; Kaya et al., 2015).

In this paper, two bitches with an abnormal distension of the vagina (hematocolpus) were studied. It was remarkable that both bitches received an implant of deslorelin acetate in a prepubertal time. The presence of an obstruction in the genital tract caused an obstacle to normal drainage and an accumulation of fluid cranially to the stenotic point. When cycling female dogs have such obstructions the collection of fluid mixes with the typical bloody discharge of proestrus; these animals show the so-called dry heats (without bloody discharge from vulva) (Viehoff e Sjollema, 2003). Specific genes involved and heritability of developmental disorders of the genital tract have not yet been fully defined in bitches (McIntyre et al., 2010). Developmental disorder of the gonad and kidney may be associated finding, as consequence of

failure of gene expression or lack of bloody supply in the genital segment (McIntyre et al., 2010). Some causes may even be acquired after birth, since the development of the internal and external genitalia is completed by puberty. There was a reasonable suspicion that the treatment received by prepubertal bitches contributed to the determinism of the lesion. The treatment applied at prepubertal time is able to arrest the development of the genital tract throughout the period of treatment (Marino et al., 2014). In some female dogs, these changes are probably not completely reversible at vaginal level.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Case report
Κλινικό περιστατικό

# Arthrectomy for traumatic proximal interphalangeal arthritis in the lateral digit in a heifer

**A. Goto[1], N. Tsuzuki[2], A. Kajihara[1], T. Moriyama[1], Y. Sasaki[1], N. Zushi[1], K. Hatate[3], K. Iino[2], N. Yamagishi[2,3]**

[1] *Veterinary Medical Center, Obihiro University of Agriculture and Veterinary Medicine, Japan*

[2] *Section of Large Animal Clinical Sciences Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Japan*

[3] *The United Graduate School of Veterinary Sciences, Gifu University, Japan*

**ABSTRACT.** Instructive information on the treatment for chronic deep infections of digital joints in a 9-month-old Holstein heifer is described in this report. Preoperative ultrasonographic and radiographic examinations revealed soft tissue swelling and subchondral bone lysis at the distal part of the proximal phalanx in the lateral digit. Arthrectomy was performed under xylazine sedation to remove infectious articular cartilage tissues. Immature callus formation was observed via radiography at the surgical site by the 28th postoperative day. On the 48$^{th}$ postoperative day, callus fell into disrepair on the radiographs along with aggravation of the locomotion score. After the application of a half-limb cast, the immature callus formed again by the 62nd postoperative day (11 months), and bony callus formation was observed by the 74th postoperative day. Thereafter, the heifer could walk well with marked improvement in the locomotion score. The withers height of the heifer at 13 months (136 cm) was within the range of that in control heifers of the same age on this farm (133 ± 3 cm); however, the body weight (BW) of this heifer (322 kg) was lower than the BW of controls (384 ± 26 kg). The BW gain from 11 to 13 months of age seemed to be higher in the present heifer (+76 kg) than in controls (+55±20 kg), suggesting that BW of the present heifer was returning to the original BW. Based on these observations, we suggested that arthrectomy was an effective treatment option for the present case of digital joint arthritis.

***Keywords:*** arthrectomy, arthritis, cow, external fixation, productivity

*Corresponding Author:*
Nao Tsuzuki, 2-11 Inada, Nishi, Obihiro, Hokkaido 080-8555, Japan
E-mail address: tsuzuki@obihiro.ac.jp

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## CASE HISTORY

A 9-month-old Holstein heifer (228 kg body weight; BW) was referred to the Obihiro University Veterinary Medical Center owing to a traumatic lesion on the proximal interphalangeal joint of the left hind limb that was injured 12 days prior. Before arriving to the medical center, the heifer underwent treatment with antibiotics and non-steroidal anti-inflammatory drugs by the referring veterinarians. Physical examination at the day of admission revealed a rectal temperature of 39.1°C, heart rate of 144 bpm, and respiratory rate of 72 bpm. The heifer had a non-weight-bearing left hind limb and marked pain and swelling around the proximal interphalangeal joint. A locomotion scoring system scored on a scale of 1 to 5 (Sprecher et al., 1997) gave a score of 5 that declared the most severe lameness. Several abrasions were observed on the skin around the proximal interphalangeal joint; one of those formed a 5-cm-deep open wound with suppurative exudate at the cranial side of the lateral proximal interphalangeal joint (Figure 1A). Preoperative radiographs confirmed soft tissue swelling and subchondral bone lysis at the distal part of the proximal phalanx in the lateral digit of the affected limb, suggesting proximal interphalangeal arthritis (Figure 1B). Ultrasonography of the lateral digit of the left hind limb revealed that the open wound was connected to the lateral proximal interphalangeal joint while conserving the superficial and deep digital flexor tendon structures (Figure 2).

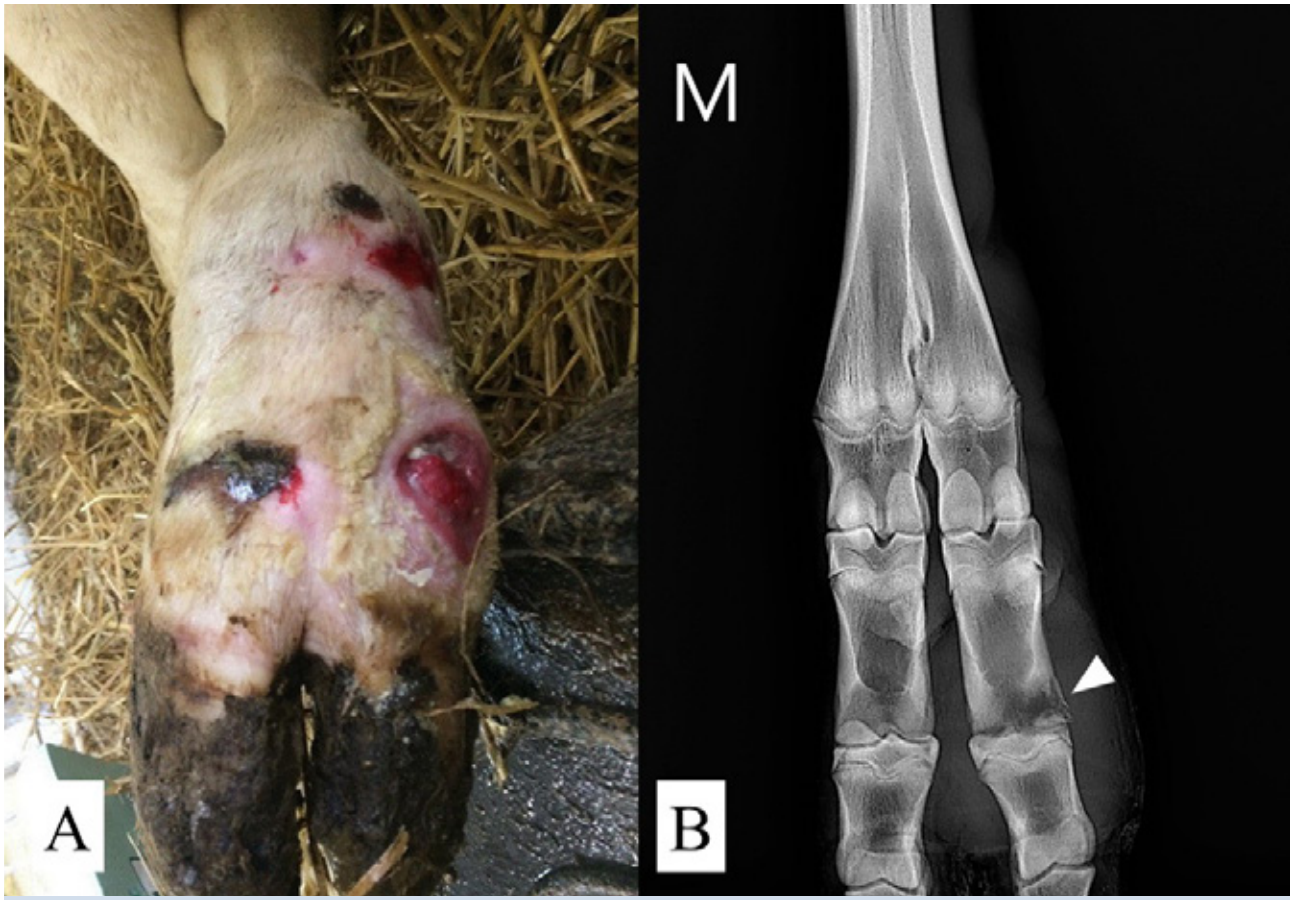


**Fig 1.** Pre-surgical photograph and radiograph (Dorsal-Plantar view) of the distal left hind limb. (A) Site of trauma around the proximal interphalangeal joint. (B) Subchondral bone lysis at the proximal interphalangeal joint in the lateral digit of the affected limb (white arrow). Swelling of soft tissue is recognized at the circumference of joint.

**Fig 2.** Ultrasonography image of the lateral digit of the left hind limb. Open wound was connected to the lateral proximal interphalangeal joint (white arrow).

The heifer was sedated with 0.1 mg/kg BW of xylazine (Seractal; Bayer, Osaka, Japan) intravenously and ceftiofur (Excenel; Zoetis, Tokyo, Japan; 2 mg/kg BW) was administered intramuscularly. The animal was positioned in the right lateral recumbency with the left hind limb upward. The skin around the proximal interphalangeal joint was aseptically prepared, the sciatic nerve blockade under ultrasound-guidance (Re., et al 2014) and local infiltration anesthesia around the open wound at the lateral proximal interphalangeal joint were induced by injecting of 2% lidocaine solution (Xylocaine; AstraZeneca, Osaka, Japan).

First, the degenerative cutaneous and subcutaneous tissues were debrided from the open wound using a surgical scalpel, and communication into the proximal interphalangeal joint was confirmed. Next, artherectomy was performed using a bone rongeur and an osteotrite to remove infectious articular cartilage tissues. The surgical wound, including the crevice of artherectomy, was then irrigated with 3 liters of sterile saline using an infusion tube that was 2.1 mm in diameter. Finally, the surgical wound was bandaged to cover the lateral digit, and hoof block was applied on the heel of the medial digit of the affected limb for relieve a load applied to a lateral digit. The surgery was completed in 40 minutes after xylazine administration, and intravenous atipamezole (0.01 mg/kg BW; atipame-chu; Kyoritsu Pharmaceutical, Tokyo, Japan) was administered to the heifer to reverse the effect of sedation.

After complete recovery from sedation, the heifer returned to the farm. Ceftiofur (2 mg/kg BW, intramuscularly, SID) was administered for 5 days postoperatively, and the bandage was changed after lavage was performed at intervals of 2 days. Hoof block was removed 14 days after surgery.

On the 28th postoperative day, radiographic examination was conducted, which revealed an immature callus at the distal part of the proximal phalanx of the lateral digit with reduced adjacent soft tissue swelling in the affected limb (Figure 3). Results of ultrasonography confirmed that the swelling of the soft tissues around the surgical wound subsided. The locomotion score improved (a score of 3) on that day.

**Fig 3.** Post-surgical radiograph (Dorsal-Plantar view) of the distal left hind limb obtained on the 28th postoperative day. An immature callus was observed on the lateral side of proximal phalanx (white arrow). Soft tissue swelling was still recognized at the circumference of joint.

On the 48th postoperative day, the locomotion score aggravated (a score of 4) although the surgical wound was closed without swelling (Figure 4A). Radiography confirmed disrepair of the immature callus at the distal part of the proximal phalanx in the lateral digit of the affected limb (Figure 4B). An external coaptation with a half-limb cast was applied from the sole of the hoof to the mid-metacarpus proximally (Figure 4C).

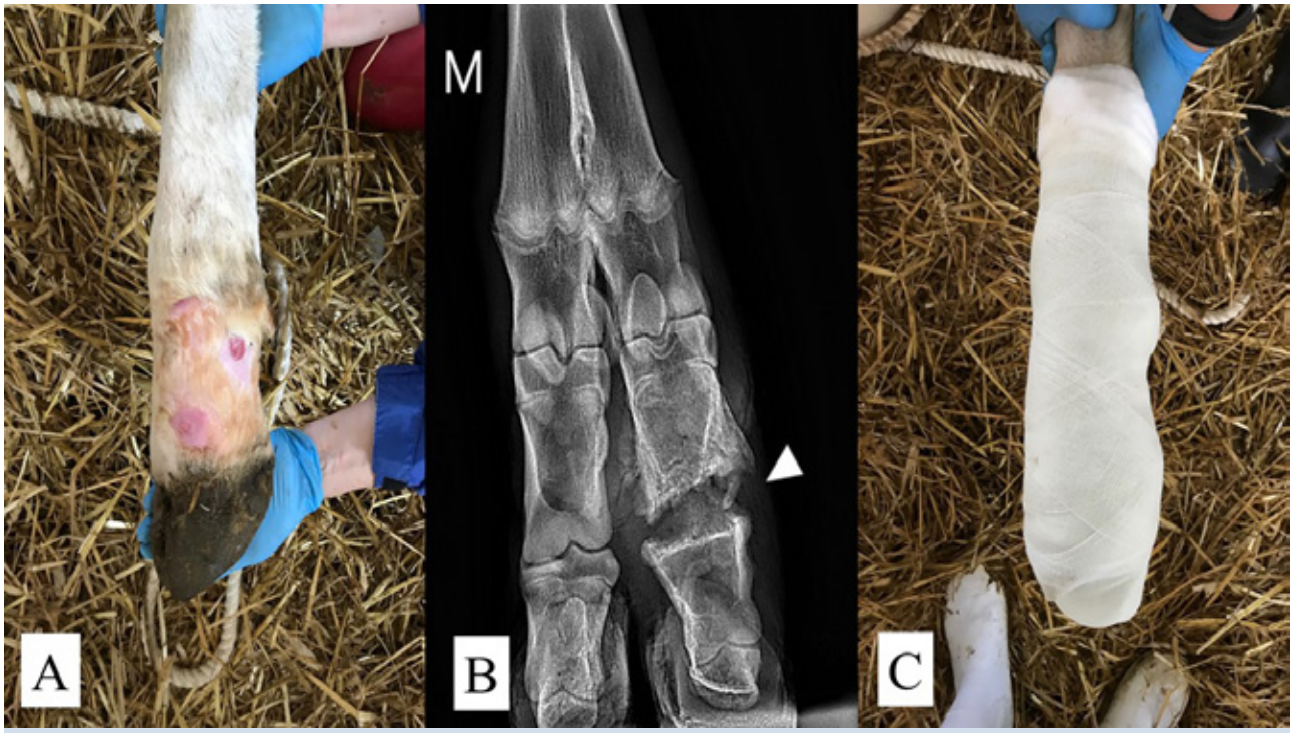


**Fig 4.** Post-surgical photograph and radiograph (Dorsal-Plantar view) of the distal left hind limb on the 48th postoperative day. (A) Well-healed wound. (B) Disrepair of immature callus at the distal part of the proximal phalanx in the lateral digit of the affected limb (white arrow). Reduced soft tissue irritation recognized at the circumference of joint. (C) An external coaptation with a half-limb fiberglass cast.

On the 62nd postoperative day, the immature callus was observed again at the distal part of the proximal phalanx on the radiographs (Figure 5A), and the locomotion score had improved (a score of 3). The half-limb cast was exchanged on that day.

On the 74th postoperative day, radiography was performed, which revealed bony callus formation at the distal part of the proximal phalanx (Figure 5B). The heifer was able to walk better with improvement in the locomotion score (a score of 2); the half-limb cast was then removed.

On the 88th postoperative day, the gait of the heifer seemed to be normal; the locomotion score was 1.

At 6 months post-surgery, there were no abnormalities in the stride and weight shift of the gait, although the heifer showed a cowhocked posture when standing (Figure 6A and B). Radiography was performed, which revealed adequate bony callus at the distal part of the proximal phalanx in the lateral digit of the affected limb (Figure 6C). The withers height of the heifer was 136 cm (age: 13 months), which was comparable to that of the control (25 heifers of the same age in this herd) heifers (average: 133 ± 3 cm). Figure 7 shows the change in BW of the present heifer and those of control from 1 to 13 months based on the records from this farm. BW of the heifer was 266 kg approximately 2 weeks before the injury occurred, which reduced to 228 kg (−38 kg) prior to arrival to our hospital (age: 9 months). After arthrectomy was performed, BW of the heifer was 246 kg at 11 months, which was increased to 322 kg (+76 kg) at 13 months. The mean BW of the control was 329 kg at 11 months and increased to 384 kg (+55 kg) at 13 months.

**Fig 5.** Post-surgical radiograph (Dorsal-Plantar view). An immature callus was observed on the lateral side of proximal phalanx again (white arrow) on (A) the 62th and (B) the 74th postoperative days.

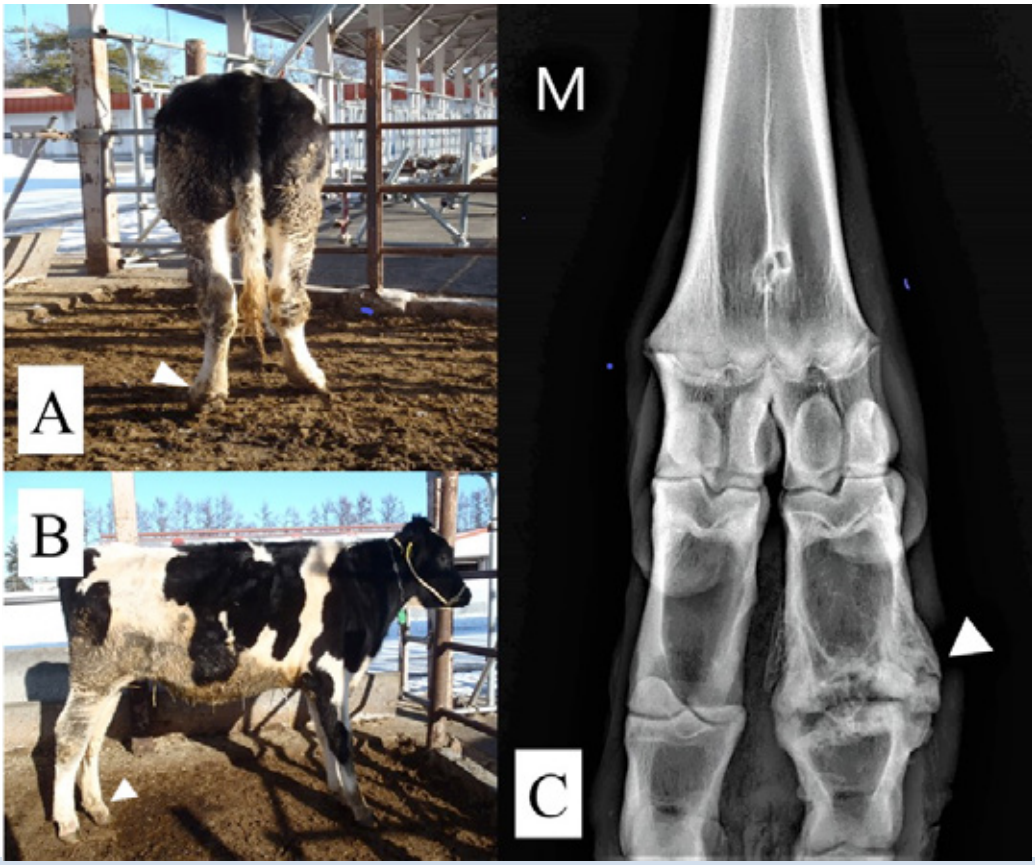


**Fig 6.** Post-surgical photograph (Dorsal-Plantar view) and radiograph in the 6th postoperative month. (A) Sufficient weight-bearing on the affected limb of dorsal view (white arrow). (B) Sufficient weight-bearing on the affected limb of lateral view (white arrow). (C) Radiographs confirmed an adequate bony callus at the distal part of the proximal phalanx in the lateral digit of the affected limb (white arrow).

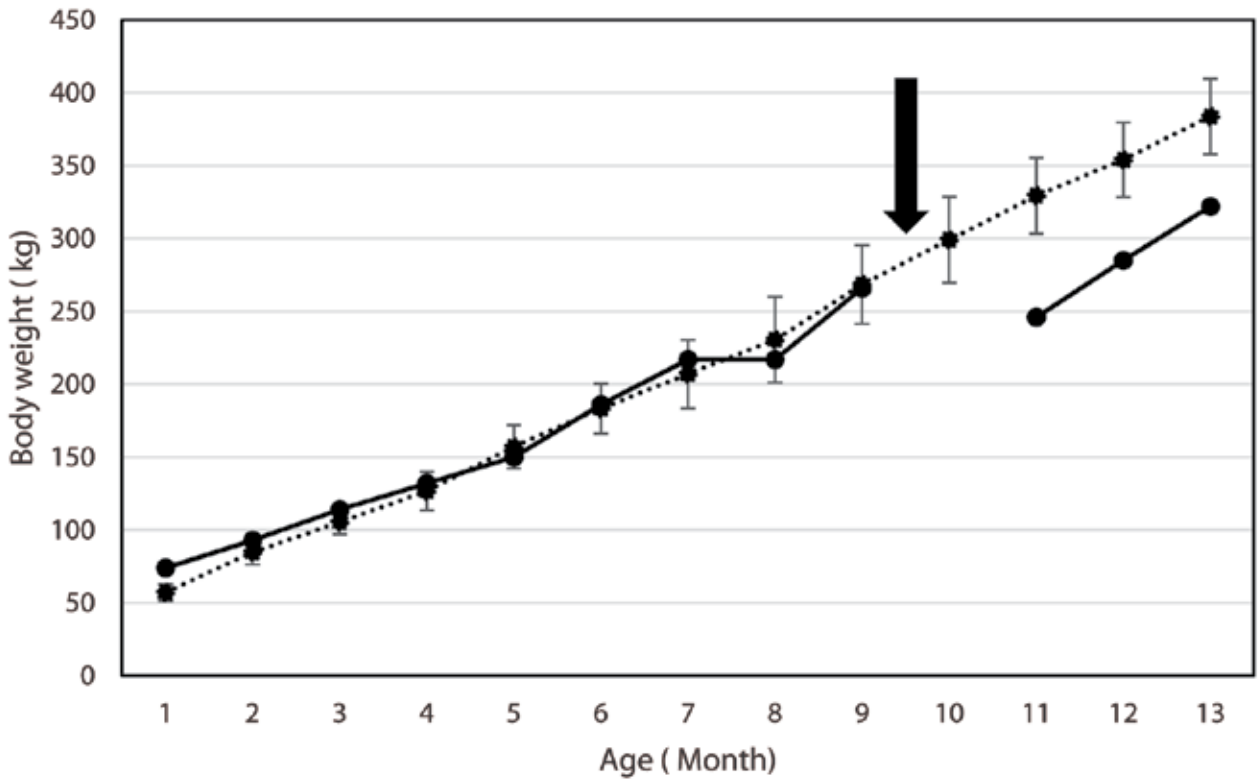


**Fig 7.** Change in the body weight of the present heifer (solid line) and control heifers (n=25) in this herd (dotted line; mean ± standard deviation) from 1 to 13 months of age. The black arrow indicates the time of injury in the heifer.

## DISCUSSION

For 12 days prior to arrival, the present heifer had a non-weight-bearing left hind limb with marked pain and swelling around its proximal interphalangeal joint, despite receiving treatment with antibiotics and non-steroidal anti-inflammatory drugs by the referring veterinarians. We decided to perform arthrectomy, followed by synarthrosis. The heifer had a favorable clinical and radiographic outcome with good wound healing; however, repeated surgical wound lavage followed by external coaptation was needed up to the 74th postoperative day.

Septic arthritis is the most common condition affecting joints of cattle. It can be caused by direct trauma, an adjacent infection, or systemic infection (Desrochers, 2004). Once pus accumulates in the joint cavity and invades the peri-articular soft tissues, needle lavage and arthrotomy with open drainage are insufficient to resolve the infection (Van Huffel et al., 1989; Starke et al., 2006). When bones are infected, surgical debridement of the devitalized or irreparably damaged bone is especially critical (Orsini, 2017). For interphalangeal joint septic arthritis, reported therapeutic options include conservative management, digit amputation, facilitated arthrodesis, and fenestration of the wound (Lewis et al., 2009). In this case, we decided that conservative management was inadequate to control the infection owing to extension of deep infection into the bones and/or periarticular soft tissues in the radiographs. In addition, preoperative radiography revealed subchondral bone lysis, suggesting the presence of a chronic bone infection. Chronic osteomyelitis with persistent drainage and sequestrum formation is resistant to eradication by long-term antibiotics alone; therefore, surgical intervention is the only effective method to eliminate such an infection and promote healing (Johnson and Buckley, 2007).

Digit amputation and digit arthrodesis surgery have been performed to successfully treat deep infections to the digits in cattle (Pesja et al., 1993; Desrochers et al., 1995). Digit arthrodesis surgery was superior to digit amputation with regards to longevity and productivity of cattle during the postoperative days (Jean and Desrochers., 2004; Bicalho et al., 2006). Conversely, disadvantages of arthrodesis of the interphalangeal joint in comparison to digit amputation are that it is more expensive, technically demanding,

and requires more postoperative care. Cattle also have a slower return to previous production following arthrodesis owing to the length of the procedure and the pain it causes (St-Jean and Desrochers., 2004). Based on this comparison, we opted for arthrectomy to treat digital joint arthritis of the heifer.

On the 48th postoperative day, the surgical wound seemed to be healing, but the locomotion score remained aggravated. Then, external coaptation with a half-limb cast was applied because disrepair of immature callus at the lateral proximal interphalangeal joint was observed on radiography. Excessive interfragmentary instability will impedes cartilage replacement, diminishes angiogenesis, and prevents bone from bridging the fracture gap (Einhorn and Gerstenfeld, 2015). In this case, improvement of the postoperative condition at the surgical site during the first month was likely to allow weight bearing of the left hind limb, resulting in an excessive interfragmentary instability and injury of the immature callus at that joint. Half-limb casts can be used for immobilization of phalangeal fractures (Anderson and Jean 2008) and can provide substantial stability to induce bone union (El-Shafaey et al., 2014). Therefore, a cast with a fenestration on the surgical site should have been applied from the beginning of the postoperative treatment in this heifer. Alternatively, it has been reported that stabilisation of the joint and the removal of the load on it by means of a steel-reinforced synthetic resin is important for the healing process (Starke et al., 2006). Moreover, it has been reported that use of the screw system in cattle is an excellent treatment option for distal interphalangeal joint arthrodesis with minimal postoperative morbidity and excellent return to function (Lewis et al., 2009).

Although the withers height of the present heifer was within the average of that of control heifers on this farm, BW of the heifers at 13 months (322 kg) was still lower than the BW of the control heifers (384 ± 26 kg). However, the BW gain for 2 months (between 11 to 13 months) seemed to be higher in the present heifer (+76 kg) than in control (+55 ± 20 kg), suggesting that BW of the present heifer was returning to the original BW. Satisfactory functioning of the reproductive organs (ovary and uterus) was observed in this heifer; the owner is planning to breed the animal. Based on these observations, we suggested that arthrectomy was an effective treatment option for digital joint arthritis in the present case for better expected recovery of productivity.

In the early infection stage of arthritis, the accuracy of the diagnosis may be reduced because radiographic abnormalities are often difficult to detect (Kofler, 2009). Ultrasound examination can be useful for detecting soft tissue damage around distal limbs (*esp*, tendinitis and abscess) of cattle (Kofler and Edinger, 1995). Therefore, in this case, it will be possible to diagnose degrees of inflammation and bone infection by combined use of radiographic and ultrasonographic examinations, resulting in the possibility of shortening the duration of postoperative treatments. Furthermore, use of half-limb cast with fenestration or a screw system were likely to shorten the duration of postoperative treatment. Hence, the current case report provides instructive information on the treatment for chronic deep infections of bovine digital joints. Improved strategies for diagnosis and surgical wound management are required for similar cases of digital joint arthritis.

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Case report
Κλινικό περιστατικό

# Conjoined Twins in Red Sokoto Goat

**I. Ahmad[1,*], U.M. Chafe[2], A.A. Umar[2], M.D. Salisu[3]**

[1]*Directorate of Animal Health and Livestock Development, Gusau, Zamfara Nigeria*

[2]*Faculty of Veterinary Medicine, Usmanu Danfodiyo University Sokoto, Nigeria*

[3]*National Animal Production Research Institute, Ahmadu Bello University Zaria, Nigeria*

**Abstract.** A case of female conjoined twins was found in Red Sokoto goat, delivered alive along with a free male kid without obstetrical assistance. The abnormal twins were examined clinically and at postmortem. Based on morphological features, they could be classified as thoraco-omphalopagus symmetrical conjoined twins. Autopsy showed that fusion occurred at ventral-midline from the cranial region of the thorax to abdomen caudal to the umbilicus; thus, only head, neck and pelvis were separated. Genetic factors could be suspected in this case. This is the first report of thoraco-omphalopagus symmetrical twinned goat in Nigeria.

***Keywords**:* Conjoined twins; Red Sokoto goat; Nigeria

*Corresponding Author:*
Ibrahim Ahmad, Directorate of Animal Health and Livestock Development, Gusau, Zamfara Nigeria
Email address: ibrahimahmaad22@gmail.com

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## INTRODUCTION

Conjoined twins represent rare congenital malformations of monozygotic twins, thus abnormalities in their anatomy arise during prenatal development (Kulawik *et al*., 2017). The term duplication, often used regarding conjoined twins, does not intrinsically imply either fission or fusion process, but it describes the formation of double structures regardless of their origin (Schneevoigt *et al*., 2014). Congenital duplication etiopathogenesis is considered to be a complex of processes with various influencing factors at different time-points (Elnady and Sora, 2009). Previous report has attributed the development of conjoined twins to either environmental or genetic factors, or both (Kulawik *et al*., 2017).

Although there is paucity of information regarding the definitive cause(s) of embryonic duplications, the separation of fused twins only becomes feasible when the two components do not share any vital parts or organs. Given the developmental disorders that may affect function of systems or organs in the malformed twins, surgical separation often leads to poor prognoses and is not always an option (Kulawik *et al*., 2017). Conjoined dysmorphologies can affect several systems not only limited to the fused parts, which usually have syndromic manifestations (Binanti and Riccaboni, 2012). Organ dysfunction, failure, and even death are the implications often associated with abnormalities arising during prenatal development (Kulawik *et al*., 2017). The occurrence of conjoined twins is sporadic and very rare, thus most of its aspects remain hypothetical; however, the description of its cases benefits both veterinary and medical sciences (Schneevoigt *et al*., 2014). In domestic animals, such aberrations are rare and remain under-reported (Samuel *et al*., 2014). The present article describes a case of thoraco-omphalopagus symmetrical conjoined female twins in Red Sokoto goat.

## CASE HISTORY

On March 4, 2018, female conjoined kids weighing approximately 2.8 kg were delivered alive and without obstetrical assistance by a multiparus doe in Tsafe town, Zamfara State, Northwest Nigeria. They were delivered along with a normal male kid (1.3 kg) (Figure 1). The normal weight at birth for kids of Red Sokoto goat is about 1.5-2.0 kg. One of the conjoined kids died at the flock a day after the delivery, although it remained attached to the living twin and were referred to the Zonal Veterinary Clinic Gusau, Zamfara State (Figure 2). The 4 years old doe was managed on free range system with three other goats and a buck and had two sets of normal twin kids in the previous parturitions. Flock history revealed no evidence of use of drugs known to cause congenital defects, occurrence of teratogenic infections, or previous record of malformation.

The conjoined kids was attached from thorax to the caudal umbilicus (Figure 3). Clinical examination revealed the following: (a) two components referring to the left and right twins, named kid A and kid B (Figure 2), (b) an increased respiration rate and mild dehydration in kid B that was alive, (c) complete duplication of the head and neck down to the thorax, (d) complete duplication of the vertebral column (thoracic, lumber and sacral vertebrae), anus, vulva, and tail (e) two forelimbs attached on each kid in normal spatial orientation, (f) shared umbilical cord, and (g) two unattached pelvis with two hind limbs on each twin at normal positions. The length of the body parts for each kid was recorded (Table 1). The alive twin died during surgical separation.

**Table 1:** Body measurements of normal kid and thoraco-omphalopagus conjoined twins in Red Sokoto goat.

| | Normal kid | Left twin (A) | Right twin (B) |
|---|---|---|---|
| Head-tail length (cm) | 37.5 | 37.5 | 37.5 |
| Neck length (cm) | 2 | 2 | 2 |
| Tail length (cm) | 2 | 2 | 2 |
| Fore-and-hind limbs length (cm) | 18 | 18 | 18 |

**Figure 1.** Normal male kid.

**Figure 2.** Conjoined kids, showing two components referring to the left and right twin, named kid A and kid B.

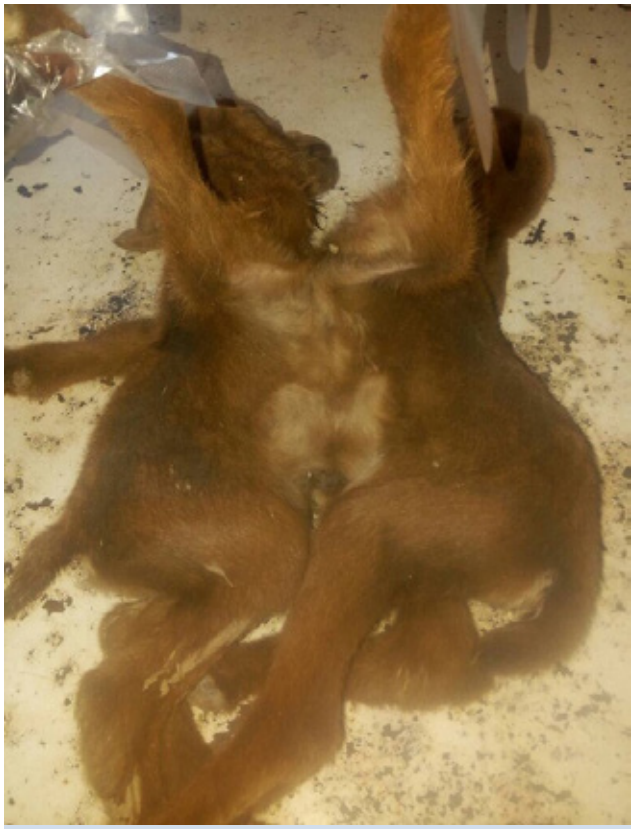

**Figure 3.** Lateral view of the conjoined twins showing fusion of thoraces and abdomen to point of umbilicus.

Autopsy revealed complete fusion of the thoraces and abdomen to the caudal umbilicus at ventral midline, two pairs of normal rib cages ventrally joined by 2 sterna, two completely divided but attached thoracic cavities containing only one hypertrophic heart (located only in kid B with two branched aorta emanating from the left ventricle; each branch supplies the organs of the individual twin component), and complete bilateral duplication of the well aerated lungs (Figure 4). The ribs formed the lateral walls of the two thoraces, articulated with the facets of thoracic vertebrae on the median plane and ventrally to the sternum. The attached thoraces were separated from the abdomen by a single diaphragm. In the abdominal cavity of each twin, the gastrointestinal tract was duplicated containing esophagus, four compartment stomachs (rumen, reticulum, omasum and abomasum), spleen, pancreas and intestines (duodenum, jejunum, ileum, caecum, colon and rectum); however, a single enlarged liver and a gall bladder were found only in kid B. Each twin contained a normal urogenital system in the characteristic anatomical positions (Figure 5). There were uterus, two kidneys, ureters and bladder that discharged into a urethra, duplicates of anus and vulva on each component.

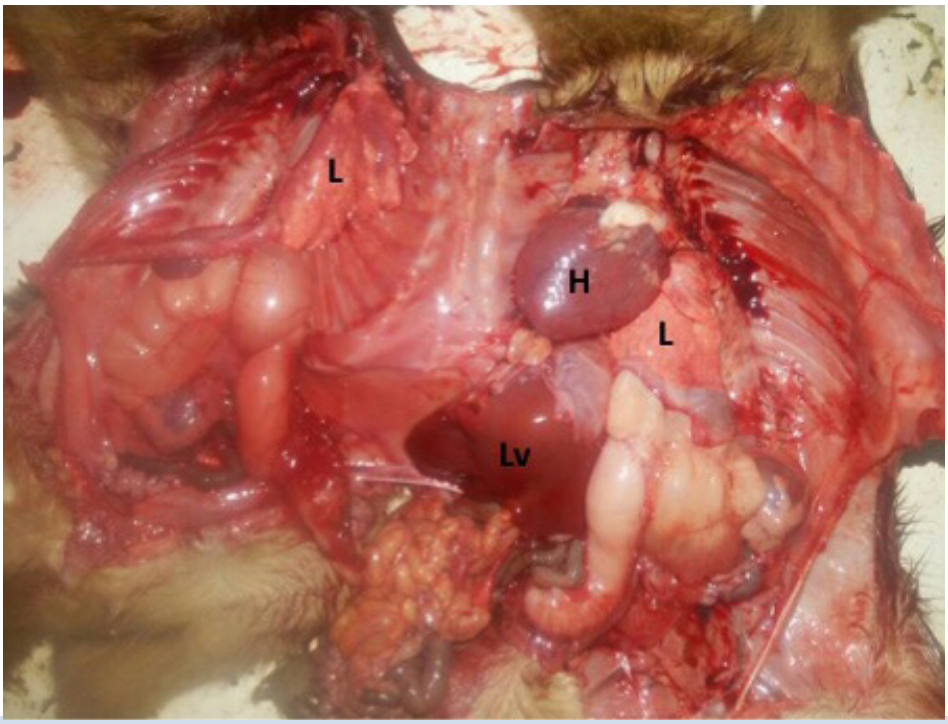


**Figure 4.** Cut opened conjoined twins showing single enlarged heart (H) and liver (Lv) found only in kid B, and bilateral duplication of the well aerated lungs (L).

**Figure 5.** Photograph showing a comparison of the internal organs recovered from each twin. 1. Uterus 2. Single heart 3. Inflated lungs 4. Kidneys 5. Intestines

## DISCUSSION

Identification of the conjoined twins is often made based on the morphological appearance of the duplication anomaly. Classifications can be based on site of the union, embryological development, anatomy, and symmetry level of the twins (Chen, 2012). Although the ventrally fused twins in this case were of equal size, they were conjoined at the thoracic cavity and cranial one third of the abdominal cavity. Kid A (parasite) has all internal organs except heart, liver, and gallbladder which makes it dependent on kid B (autosite). These kids are completely well developed and symmetrical, each exhibiting a set of structures that is an imitation of its counterpart; thus they are called diplopagus. Classification into either conjoined symmetrical (complete) or conjoined asymmetrical (incomplete) is based on the normal-length duplication of vertebral column; duplicated partially in asymmetrical and fully in symmetrical congenital twins. The externally visible point of attachment is also commonly used to classify conjoined twins. In the study subjects, this duplication occurred across the entire body parts but fused at the ventral-midline between thoraces (thoracopagus) and caudal umbilicus (omphalopagus), leading to a classification as thoraco-omphalopagus. The conjoined twins documented

in this report were similar to that previously found in goats by Binanti and Riccaboni (2012); however, this occurrence is rare (Elnady and Sora, 2009). The present case is unique because all structures are nearly doubled except heart, liver, gallbladder, and umbilicus; thus it is different from the previous report (Binanti and Riccaboni, 2012) which described additional uncommon malformations such as persistent right aortic arc, foramen ovale, and patent ductus arteriosus. Conjoined twins was female, as in previous reports (Binanti and Riccaboni, 2012; Mazaheri *et al*., 2014; Schneevoigt *et al*., 2014).

The fusion of the thorax and abdomen represents an abnormal articulation at the ventral midline, presenting morphological alterations of the sternum and abdominal wall. No obvious abnormality associated with the respiratory organs in both the thoraces was observed, as reported previously (Binanti and Riccaboni, 2012; Schneevoigt *et al*., 2014). A common liver and umbilical cord shared between the twins found in this case is similar to the report of Binanti and Riccaboni (2012). Abdominal structures such as esophagus, four chambered stomachs, intestines, pancreas and spleen were entirely duplicated without any fusion. This observation varies with the findings in the literature (Binanti and Riccaboni, 2012; Schneevoigt *et al*., 2014). The entire gastroinstestinal tract is rarely doubled in siamese twins (Spencer, 2000). The urogenital organs found in this case were doubled (four kidneys, two bladders, four ovaries, and oviducts, as well as two bicornuate uterus and vagina), as widely confirmed previously in conjoined twins (Spencer, 2000). As concern the cardiovascular system defect involved there was a single functioning heart for both kids, which is similar to heart defects previously described in congenital twinning (Binanti and Riccaboni, 2012; Schneevoigt *et al*., 2014; Kulawik *et al*., 2017). The enlarged heart and branched aorta found in the right kid B, reflected the right twin heart being the pumping donor to the left twin A. Thus, in this form of defect Twin-Twin Transfusion Syndrome (TTTS) might be a conceivable consequence of arteriovenous anastomoses (Bahlmann, 2009). Cells are not split equally in monozygotic twinning process, resulting in a larger number of cells in one twin leading to a delay in cardiac development of the twin that had received fewer cells (Benirschke, 2009).

Information about the cause(s) of congenital duplication anomalies is rarely available (Shojaei *et al*., 2012). Two hypotheses such as fusion and fission have been proposed regarding conjoined twins, but the mechanism of its development is incompletely understood (Binanti and Riccaboni, 2012). Thus, diplopagus could arise either due to incomplete separation of a single fertilized ovum as in monozygotic twins or by secondary fusion between two different embryonic axes. Mis-expression of gene and secreted protein signals may be implicated in congenital duplication, affecting the regulation of right-left asymmetry or left-right axis formation (Mazaheri *et al*., 2014; Kulawik *et al*., 2017). In Nigeria, the predominant free-range management system favors exposure of pregnant animals to toxic plants, drinking water, and forages contaminated by various chemicals (e.g. pesticides, herbicides and inorganic fertilizers) being applied without caution. Furthermore, the practices of unregulated mining activities could lead to enhanced exposure of animals to hazardous heavy metals and radiation-induced hyperthermia. Exposure to some of these factors or their combination can serve as exogenous disruptors, thereby acting as substrate in allometric growth impairments leading to in-utero developmental errors. In addition, lack of dietary supplements and frequent use of hormone treatment are in part linked to the higher incidence of conjoined twins in animals (Schneevoigt *et al*., 2014; Kulawik *et al*., 2017). Previous report described the above mentioned as probable causes of congenital abnormalities in animals (Kulawik *et al*., 2017). Moreover, a genetic mutation was speculated as the probable cause of a similar malformation described by Binanti and Riccaboni (2012).

This is the first report of thoraco-omphalopagus symmetrical conjoined twins in animals in Nigeria. The article presented the anatomical findings of conjoined twins born alive, after clinical and postmortem examinations. Malformations of skeletal, cardiovascular, and digestive systems were revealed. Prompt reporting of such cases is encouraged to aid in the epidemiological surveys of malformations in animals and accurate identification of the probable cause(s) and compounding factors.

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## CONFLICT OF INTEREST

None declared.

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