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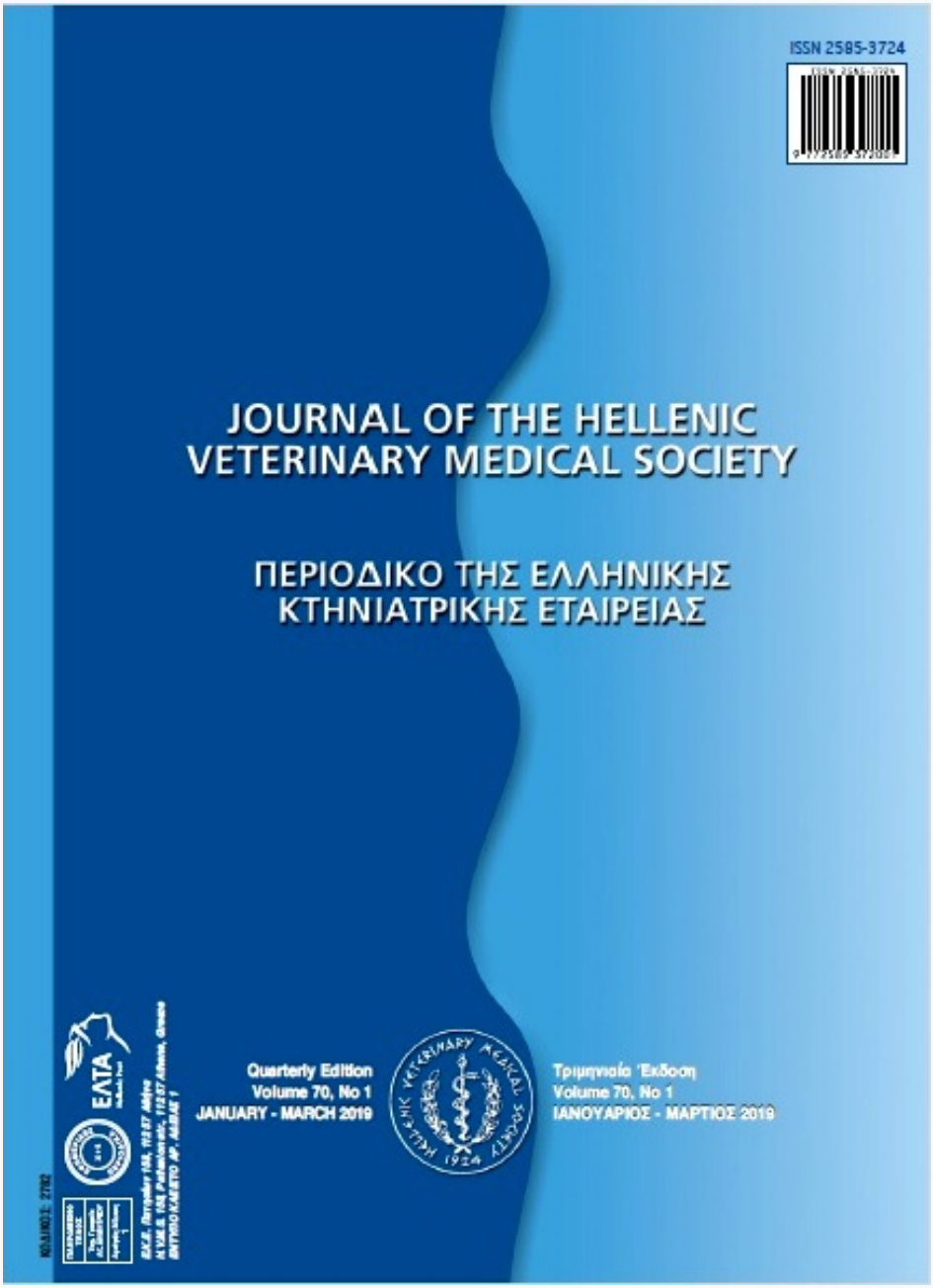

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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

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EBVS EUROPEAN BOARD OF VETERINARY SPECIALISATION

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EUROPEAN COLLEGES OF VETERINARY SPECIALISTS
ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

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1	ECAR	European College of Animal Reproduction	2
2	ECAWBM	European College of Animal Welfare and Behavioural Medicine	1
3	ECAAH	European College of Aquatic Animal Health	2
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	3
9	ECSRHM	European College of Small Ruminant Health Management	10
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
15	ECVECC	European College of Veterinary Emergency and Critical Care	0
16	ECVIM-ca	European College of Veterinary Internal Medicine--companion animals	0
17	ECVN	European College of Veterinary Neurology	0
18	ECVO	European College of Veterinary Ophthalmology	0
19	ECVP	European College of Veterinary Pathology	0
20	ECVPH	European College of Veterinary Public Health	7
21	ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22	ECZM	European College of Zoological Medicine	1
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ΕΛΛΗΝΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ ΕΤΑΙΡΕΙΑ (ΕΚΕ)
HELLENIC VETERINARY MEDICAL SOCIATY (HVMS)

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Περιεχόμενα

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 Veterinary Microbiological Institute of Votanicos, from where all members 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, Institute 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 capabilities, 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 members. 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 execution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veterinary 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 Companion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (founding member)**

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

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m^2), secretary (13m^2), lecture room (91m^2), the President's office (22m^2), the Governing Board meeting room & library (44m^2), the kitchen (18m^2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President	: Theodoros Cl. Ananiadis†
Vice-President	: Veniamin Albalas
General Secretary	: Athanassios E. Tyrpenou
Spec. Secretary	: Konstantinos Chandras
Treasurer	: Olga Sabatakou
Member	: Emmanuel Archontakis
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The Board of Directors and the Editorial Board of the Journal of the Hellenic Veterinary Medical Society, warmly thank the reviewers that substantially contributed in the successful publication of the 69th volume 2018 of the J Hellenic Vet Med Soc, the names of which are sited below in alphabetical order:

Το Διοικητικό Συμβούλιο και η Συντακτική Επιτροπή του Περιοδικού της Ελληνικής Κτηνιατρικής Εταιρείας, ευχαριστούν θερμά τους κριτές που συνέβαλαν ουσιαστικά στην επιτυχή έκδοση του 69ου τόμου 2018 του ΠΕΚΕ, τα ονόματα των οποίων παραθέτουμε στον παρακάτω πίνακα:

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Review article
Ανασκόπηση

The genus *Campylobacter:* detection and isolation methods, species identification & typing techniques

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Το γένος *Campylobacter:* μέθοδοι ανίχνευσης και απομόνωσης, ταυτοποίηση είδους και τεχνικές τυποποίησης

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ABSTRACT. *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, poultry has been identified as a significant cause of campylobacter infection in humans. The *C. jejuni* has been found to be the predominant species isolated from poultry samples and, yet, responsible for the majority of human campylobacteriosis. *Campylobacter* spp. are small, oxidase positive, microaerophilic, curved gram-negative rods exhibiting corkscrew motility and colonize the intestinal tract of most mammalian and avian species. From its very first description in late 19th century by Theodor Escherich until nowadays, a lot of research has been carried out providing a wealth of information regarding its microbiological properties. Since novel technologies constantly emerge, increasingly advanced methods for detection, identification and typing of *Campylobacter* spp. are becoming available. The aim of this article is to review the recent bibliography on *Campylobacter* focusing, especially, on its survival and growth characteristics, the laboratory methods used for its detection and isolation from clinical, animal, environmental, and food samples, the reported methods applied for its speciation, as well as the typing systems developed for subtyping of *Campylobacter.*

Keywords: *Campylobacter* spp., detection, isolation, species identification, typing.

ΠΕΡΙΛΗΨΗ. Το *Campylobacter* είναι παγκοσμίως αναγνωρισμένο ως ο συχνότερος αιτιολογικός παράγοντας της βακτηριακής αιτιολογίας, διαρροϊκής τροφοδηλητηρίασης, ενώ τα πουλερικά έχουν αναγνωριστεί ως η κύρια αιτία μόλυνσης του ανθρώπου. Το *C. jejuni* είναι το είδος που απομονώνεται συχνότερα από δείγματα προερχόμενα από πουλερικά και συνεπώς ευθύνεται για τα περισσότερα περιστατικά ανθρώπινης καμπυλοβακτηρίωσης. Τα *Campylobacter* spp. είναι μικροί, θετικοί στη δοκιμή οξειδάσης, μικροαερόφιλοι,gram-αρνητικοί, κυρτοί βάκιλοι που παρουσιάζουν χαρακτηριστική ελικοειδή κίνηση και αποικούν τον εντερικό σωλήνα των περισσότερων θηλαστικών και πτηνών. Από την πρώτη περιγραφή τους στα τέλη του 19ου αιώνα από τον Theodor Escherich έως σήμερα, έχει διεξαχθεί σημαντική έρευνα που πρόσφερε πλούτο πληροφοριών σχετικά με τα μικροβιολογικά τους χαρακτηριστικά. Χάρη στη συνεχή εμφάνιση καινοτόμων τεχνολογιών, όλο και πιο προηγμένες μέθοδοι ανίχνευσης, ταυτοποίησης και γενοτύπησης γίνονται διαθέσιμες. Σκοπός αυτού του άρθρου είναι η ανασκόπηση της πρόσφατη βιβλιογραφία σχετικά με το *Campylobacter* εστιάζοντας κυρίως στα καλλιεργητικά του χαρακτηριστικά, τις εργαστηριακές μεθόδους που χρησιμοποιούνται για την ανίχνευση και την απομόνωσή του από κλινικά, ζωικά, περιβαλλοντικά και δείγματα τροφίμων, τις καταγεγραμμένες μεθόδους που χρησιμοποιούνται για τη ταυτοποίηση του είδους, καθώς και τα συστήματα γενοτύπησης που έχουν αναπτυχθεί για την υποτυποποίηση του *Campylobacter*.

Λέξεις ευρετηρίασης: Campylobacter spp., τροφιμογενή παθογόνα, Ελλάδα, πτηνά, επιπολασμός, παράγοντες κινδύνου.

INTRODUCTION

Campylobacters are ubiquitous bacteria, able to colonize mucosal surfaces, usually the intestinal tract of most mammalian and avian species tested (OIE, 2008). *Campylobacter* is well recognized as the leading cause of bacterial foodborne diarrheal disease worldwide; while, the poultry has been identified as a significant source for *Campylobacter* infections in humans. The *C. jejuni* is the predominant species isolated from poultry samples, followed by *C. coli,* and other less-detected *Campylobacter* species such as *C. lari* (EFSA, 2010). The *C. jejuni* is considered responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by C. lari (Zhang and Sahin, 2013). The incidence of human campylobacteriosis has been steadily rising worldwide since 1990's (WHO, 2011). While in Greece there is a dearth of data (Natsos et al., 2016), in the European Union, campylobacteriosis has been the most commonly reported zoonosis since 2005 (EFSA, 2006; EFSA and ECDC, 2017), in the United States, the incidence of Campylobacter infections per 100,000 people was the highest along with Salmonella (CDC, 2018), in Australia *Campylobacter* has been found to be the most common cause of acute bacterial diarrhea among all

the notified enteric pathogens (Stafford, 2010), while human campylobacteriosis is hyperendemic in many developing areas of the world (Coker et al., 2002).

THE GENUS Campylobacter: A HISTORICAL OVERVIEW

The generic name *Campylobacter*, from the Greek kampylos (curved) and baktron (rod), was given by Sebald and Véron (1963) to the group of bacteria formerly known as the microaerophilic vibrios, due to their special *characteristics* (Moore et al., 2005). It is believed that Campylobacter species were first described by Escherich (1885) who observed non-culturable spiral-shaped bacteria in the large-intestinal mucus of infants who had died of cholera infantum (Vandamme, 2000), while McFadyean and Stockman (1913) were the first to isolate these organisms from the uterine exudate of aborting sheep. A few years later, the study of Butzler et al. (1973) raised the interest in *Campylobacter* as a cause of human disease by noting their high incidence in cases of diarrhea. The first successful isolation of Campylobacter from human faeces had been accomplished one year before by using a filtration technique (Dekeyser et al., 1972). Later, the isolation of Campylobacter became a routine in the field of clinical microbiology and Campylobacter spp. rapidly became recognized as a common cause of bacterial gastroenteritis (Fitzgerald et al., 2008a).

CLASSIFICATION

In the 1970s, there was much confusion over *Campylobacter* nomenclature (Skirrow, 1994); however, the classification of Véron and Chatelain (1973) forms the basis of currently approved nomenclature. The family Campylobacteraceae, proposed by Vandamme and De Ley (1991), consists of two genera, *Campylobacter* and Arcobacter (Vandamme, 2000); while, the genus of *Campylobacter* currently contains 34 species and 14 subspecies (Parte, 2014). The taxonomy of the Campylobacter genus, which has been revised many times (Debruyne et al., 2008), is reviewed by On (2001).

MORPHOLOGY

Members of the *Campylobacter* genus are slender, spirally-curved, and non-sporeforming gram-negative rods. The size of the cells is small and ranges from 0.2 to 0.9 μm in width and 0.5 to 5 μm in length (Silva et al., 2011). Some species, such as *C. hominis* and *C. gracilis,* form straight rods (Fitzgerald et al., 2008a). Most species are motile by means of a single polar unsheathed flagellum inserted at one or both poles of the cells (monotrichate or amphitrichate) (Vandenberg et al., 2005). The only exceptions are *C. showae*, which has up to five unipolar flagella, and C. gracilis, which has none and is immotile (Debruyne et al., 2008). Motility is rapid and darting, with the bacteria spinning around their long axes in a corkscrew fashion (Vandenberg et al., 2005). Because of their small size and motility, *Campylobacter* spp. are able to pass through membrane filters (0.45 to 0.65 μm) with relative ease, a property used for isolating Campylobacter spp. from clinical samples (Bolton, 2000; Steele and McDermott, 1984).

GROWTH AND SURVIVAL CHARACTERISTICS

Under ideal conditions, Campylobacters produce visible growth after 24 h at 37 °C, but colonies are not well formed until 48 h; however, it may take up to 72-96 hours of incubation to observe some slow-growing strains (Corry et al., 1995). Depending on the media used, the appearance of *Campylobacter* colonies may vary. If the agar is moist, the colonies may appear gray, flat, irregular, and thinly spreading; whereas, round, convex, or glistening colonies may be formed when plates are dry (Corry et al., 1995; Vandenberg et al., 2005). Since the pathogenic *Campylobacter* species grow at 37-42 °C, with an optimum growth temperature of 41.5 °C, they are used to be referred as thermophilic Campylobacters: although Levin (2007) suggested the term “thermotolerant” since they do not exhibit true thermophily (growth at 55°C or above). Campylobacters are incapable of growth below 30°C, as they lack cold shock protein genes which play a role in low-temperature adaptation (Silva et al., 2011).

These non-spore-forming and fastidious bacteria neither ferment nor oxidize carbohydrates; instead, they obtain energy from the degradation of amino acids, or tricarboxylic acid cycle intermediates (Kelly, 2001; Vandamme, 2000). They are essentially

microaerophilic, thus an atmosphere with low oxygen tension (5% O2, 10% CO2, and 85% N2) is regarded as the most suitable for *Campylobacter's* incubation (Garénaux et al., 2008). Oxidase activity is present in all species except for *C. gracilis* (Silva et al., 2011).

Except of their fastidious growth requirements, *Campylobacter* spp. are very fragile and more susceptible than most bacteria to many environmental conditions, such as temperature and pH changes, low humidity, presence of oxygen and UV irradiation, and to many chemical agents such as disinfectants (Isohanni, 2013). *Campylobacter* spp. are easily inactivated by heat treatments with their D-value being less than 1 min (Silva et al., 2011), while freezing and thawing causes a 1-2 log10 fall in viable numbers, yet bacteria remain alive for many months at -20 oC (Vandenberg et al., 2005). Most species have a pH growth range of 5.5-8.0, though optimal growth occurs at pH 6.5-7.5 and water activity (aw) equal to 0.997 (approximately 0.5% w/v NaCl), as mentioned by Silva et al. (2011).

In some species, notably *C. jejuni* and *C. lari,* cultures that are exposed to atmospheric oxygen (Vandenberg et al., 2005) or other unfavorable conditions, such as changes in temperature and pH, dehydration and low nutrient availability, may undergo coccal transformation (Jackson et al., 2009; Kassem et al., 2013; Oliver, 2010; Rollins and Colwell, 1986), which seems to be a degenarative process in response to these circumstances (Harvey and Leach, 1998; Reezal et al., 1998). Those viable but non-cultivable cells (VBNC) have been shown to be unable to grow in subculture; even though the possibility that they can revert to spiral forms after passing through the intestinal tract of chickens or humans remains unanswered (Oliver, 2010; Vandenberg et al., 2005) and even their existence is contentious (Silva et al., 2011).

LABORATORY ISOLATION AND DETECTION METHODS

In a clinical context, a laboratory is mainly asked to detect campylobacters in the faeces of patients with diarrhea. The same purpose also applies when it comes for samples derived from animal stool, environmental materials, or processed food. There are two main categories regarding the detection method used: the conventional culture-based isolation methods and the culture-independent methods.

Culture-based isolation methods

The conventional method for isolating the common enteric *Campylobacter* species from faecal samples is a primary plating on selective media followed by incubation at 42 oC in a microaerobic atmosphere (Vandenberg et al., 2006). Though faeces often contain large numbers of viable *Campylobacter* making their detection easily possible by direct plating on selective media (Fitzgerald et al., 2008b), food products and environmental samples tend to have fewer numbers of stressed *Campylobacter* cells, thus, an enrichment step in liquid medium before plating on solid agar plates is indicated (Corry et al., 1995). Several enrichment broths (e.g. Bolton broth, *Campylobacter* enrichment broth and Preston broth), that are available to be used before plating, have been compared for their efficacy (Baylis et al., 2000).

The first selective culture medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow. Since then more than 40 solid and liquid selective culture media for culturing *Campylobacter* from clinical and food samples have been reported and evaluated (Habib et al., 2008; Kiess et al., 2010; Potturi-Venkata et al., 2007). All the selective media contain a basal media, either blood or other agents such as charcoal, to quench oxygen toxicity (Fitzgerald et al., 2008a), and a variety of combinations of antibiotics to which thermophilic *Campylobacter* species are intrinsically resistant; such antibiotics (like polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin) suppress the growth of many background microbial flora present in samples allowing the isolation of slow-growing Campylobacter spp. (Vandenberg et al., 2005; Zhang and Sahin, 2013).

The most recent standard method (ISO, 2006a) for detection and isolation, as well as a direct plating method for enumeration of *Campylobacter* spp. (ISO, 2006b), use mCCDA as the selective agar, while Bolton broth is used for the enrichment step. Alternative enrichment and plating combinations for enumeration and detection of *Campylobacter* in chicken meat have

been evaluated (Habib et al., 2011) and seem to provide significantly better results.

Direct detection methods

Microscopic observation of direct smear or wet preparation, in the case of liquid feaces, may reveal the presence of curved rods characteristic of *campylobacters* (Vandenberg et al., 2005). Dark-field microscopy may also reveal – besides the characteristic morphology – the darting motility of Campylobacter species (Fitzgerald et al., 2008a). Moreover, the direct Gram-stain with carbol-fuchsin counterstain method, though underutilized, may provide a presumptive result within 30 minutes of receipt of a feacal sample in the laboratory with relatively high sensitivity and at low cost (Wang and Murdoch, 2004).

There are also nonculture-based methods for the direct detection of campylobacters in human or animal faeces and processed food samples, which allow the identification of this fastidious organism without the specialized media and equipment needed for Campylobacter culture. Several enzyme immunoassays (EIA), which are based on antigen-antibody interaction, have been developed for this purpose in human faeces and are commercially available in a form of kits (Bessède et al., 2018; Dediste et al., 2003; Granato et al., 2010; Tolcin et al., 2000). While the culture-independent diagnostic tests (CIDTs) are convenient to use, the sensitivity, specificity, and positive predictive value of *Campylobacter* stool antigen tests have found to be highly variable (Bessède et al., 2011; Giltner et al., 2013; Granato et al., 2010) and therefore their use as standalone tests for direct detection of *Campylobacter* in stool is questioned. In addition, the utility of these assays for detection of campylobacters in chicken faeces, which represent the main reservoir of pathogenic *Campylobacter* species, remains to be determined (Zhang and Sahin, 2013). Regarding the food samples, although commercial EIAs are available for culture-independent identification of *Campylobacter* spp., these assays have not been extensively validated (Oyarzabal and Battie, 2012) and are mainly applied to enriched cultures (Bailey et al., 2008; Bohaychuk et al., 2005). Commercial and/or published immunological methods used to identify *Campylobacter* spp. in food and stool samples have been reviewed by Oyarzabal and Battie (2012).

Many PCR-based assays have been described to directly detect campylobacters in human stools from clinical cases (Al Amri et al., 2007;Lin et al., 2008; Zhang et al., 2013), feacal samples from bovine (Inglis and Kalischuk, 2004) and pigs (Jensen et al., 2005; Leblanc-Maridor et al., 2011), ceacal and feacal samples from broilers (Al Amri et al., 2007; Lund et al., 2003; Rodgers et al., 2012), samples from poultry meat (Debretsion et al., 2007; Fontanot et al., 2014; Hong et al., 2007; Josefsen et al., 2010; Schnider et al., 2010) and environmental specimens (Rothrock et al., 2009; Waage et al., 1999); although, so far these have been used only for research applications. Advantages of using a PCR approach instead of culture include same-day detection and identification of *Campylobacter* to the species level, along with the identification of the less-common Campylobacter species that are often missed by conventional culture (Kulkarni et al., 2002). However, PCR methods are more expensive and labor-intensive than culture and do not provide an isolate for further characterization such as typing and sensitivity testing.

Finally, fluorescent in situ hybridization (FISH), with the application of highly specific oligonucleotide probes, may serve for the detection and identification of thermotolerant Campylobacter spp. in feacal and liver samples, and looks promising to become a future monitoring system in a logistic poultry slaughter concept (Schmid et al., 2005).

SPECIES IDENTIFICATION

Among the Campylobacter spp. growing at 42 °C, the most frequently encountered species from samples of animal origin are *C. jejuni* and *C. coli,* however, low frequencies of other species have also been reported. Speciation is difficult because of the complex and rapidly evolving taxonomy along with the biochemical inertness of *Campylobacter* spp., and these problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group (Fitzgerald et al., 2008a).

Campylobacters are biochemically inactive compared with many other bacteria, thus, few phenotypic tests

are available to identify them to the species level. Generally, *C. jejuni* can be differentiated from other species based on the hydrolysis of hippurate as this is the only *Campylobacter* species that expresses the N-benzoylglycine amidohydrolase (hippuricase) gene, giving hippurate-positive result. However, variability in the hippurate reaction has been observed in some strains of *C. jejuni* resulting in hippurate-negative results (Denis et al., 1999; Jensen et al., 2005; Rautelin et al., 1999). Nalidixic acid and cephalothin susceptibility testing have been used in species identification in the past (Barrett et al., 1988). Both *C. jejuni* and *C. coli* grow at 42 °C and are resistant to cephalothin and cefoperazone, which are valuable agents for inclusion in selective media (Vandenberg et al., 2006). Instead, *C. upsaliensis* is sensible to cephalothin (ISO, 2006a). Nowadays sensitivity to nalidixic acid may give difficulties in interpretation (OIE, 2008) since fluoroquinolone resistant and cross-resistant to nalidixic acid *Campylobacter* species have become increasingly common with rates reported to be as high as 80% (Engberg et al., 2001), therefore, antimicrobial susceptibility tests can no longer be relied upon for the phenotypic identification of *Campylobacter* isolates (Fitzgerald et al., 2008a). More biochemical tests may be applied for species identification, such as the detection of catalase which is absent in *C. upsaliensis,* and the detection of indoxyl acetate hydrolysis which is negative in *C. lari* (ISO, 2006a); whereas, more extensive speciation schemes have been described in the literature (On, 1996; Vandamme, 2000).

Because of the difficulties and the unreliability of the phenotypic identification, several molecular methods may be used as supplementary to biochemical tests or even to replace them. A variety of DNA probes and polymerase chain reaction (PCR)-based identification assays has been described for *Campylobacter* species (On, 1996; Vandamme, 2000). Detection of species-specific sequences via PCR can be helpful, especially, in cases where the differentiation between hippuricase-negative *C. jejuni* strains and *C. coli* – which are closely related species – is needed, and the application of biochemical tests alone is inadequate (Denis et al., 1999; Persson and Olsen, 2005).

TYPING AND SUBTYPING

Classification of bacterial strains at the species or subspecies level is generally known as bacterial typing or subtyping. The main purposes of bacterial subtyping are the evaluation of taxonomy, the definition of phylogenetic relationships, the examination of evolutionary mechanisms, and the conduct of epidemiological investigations (Van Belkum et al., 2001). Moreover, the use of typing methods provides the opportunity to apply more rapid, precise, and efficient foodborne pathogen surveillance and prevention practices (Wiedmann, 2002). The ability to discriminate or subtype campylobacters below the level of species has successfully been applied to aid the epidemiological investigation of outbreaks of campylobacteriosis (French et al., 2011; Sails et al., 2003a; Wassenaar and Newell, 2000), providing information to recognize outbreaks of infection, to match cases with potential vehicles of infection and to discriminate these from unrelated strains.

Typing of *Campylobacter* is a dynamic field with older methods continually being advanced and new methodologies constantly being developed (Ross, 2009). A multitude of typing systems have been developed over the last few years, however, no single technique has been declared as universally acceptable and applicable (Sails et al., 2003a), since each one has both advantages and disadvantages. A number of criteria are used to evaluate subtyping methods to define their efficacy and efficiency: two major properties that any typing system should possess in order to be adapted for further use (ECDC, 2009). The efficacy of any typing technique can be assessed in terms of typeability, reproducibility, consistency, and power of discrimination; while, the efficiency reflects the expertise required, time consumed or rapidity of the technique, flexibility, and suitability to carry out a certain investigation (Mohan, 2011).

Typing systems are based on the idea that clonally related isolates share common characteristics which can be tested to differentiate them from unrelated isolates (Eberle and Kiess, 2012). They are broadly classified into two major categories: phenotyping – applies phenotypic methods that detect the presence or absence of biological or metabolic activities

expressed by the bacteria, and genotyping – utilizes genotypic methods that involve analysis of genetic elements based on the bacteria's DNA and RNA (Arbeit, 1995).

Phenotypic methods

The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include biotyping, serotyping, phage typing, and multilocus enzyme electrophoresis. Even though most of these methods lack discriminatory power, they are still applied and are quite efficient in characterizing foodborne bacterial pathogens (Wiedmann, 2002).

Biotyping schemes based on the identification of bacterial isolates through the expression of metabolic activities, such as colonial morphology, environmental tolerances, and biochemical reactions, can group *C. jejuni, C. coli* and *C. lari* in broad categories (Eberle and Kiess, 2012; Vandenberg et al., 2006). Biotyping is useful as a first step for epidemiological investigation as it is easy to perform, relatively inexpensive, and can quickly identify bacterial isolates for further testing, however, due to its poor reproducibility and stability, and low discriminatory power it is often combined with serotyping to make the scheme more useful (Sails et al., 2003b).

Serologic typing, or serotyping, is based on the knowledge that different strains of bacteria differ in the antigens they carry on their cellular surfaces. In serotyping, antibodies and antisera are used to detect these surface antigens, thereby, distinguishing strains by the differences in their surface structure (Arbeit, 1995; Wiedmann, 2002). There are two generally accepted and well-evaluated serotyping schemes that were developed in the 1980s for epidemiological characterization of *Campylobacter* isolates: the first one is based on the heat stable O antigens (LPS, LOS and CPS) using a passive hemagglutination technique and was described by (Penner and Hennessy, 1980), and the other one, developed by Lior et al. (1982), is based on heat labile antigens using a bacterial agglutination method. Since the two schemes are complementary, they can give good discrimination when used together even with restricted panels of antisera (Vandenberg et al., 2005).

Phagetyping was initially performed to characterize *C. jejuni* and by (Grajewski et al., 1985) and is often used as an adjunct to serotyping. Concisely, the technique utilizes a set of virulent phages on a bacterial host irrespective of any receptors for attachment. If the phages are able to attach and infect the bacterial hosts, they lyse the bacterial cells producing a characteristic lytic pattern on the cultured petri dishes, referred to as 'plaques' (Grajewski et al., 1985). Like serotyping, the usefulness of phagetyping is also limited by the occurrence of non-typeable isolates and problems with cross reactivity (Sails et al., 2003b).

In multilocus enzyme electrophoresis (MLEE), bacterial isolates are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing conditions (Wiedmann, 2002). This technique has been utilized to study the congruence between other typing schemes used for *C. jejuni,* such as multilocus sequence typing (MLST) and pulse field gel electrophoresis (PFGE) (Sails et al., 2003b). Because of its limitations, MLEE has been rendered unsuitable for regular typing and has been superseded by a nucleotide-based technique, MLST, which essentially mimics the MLEE's multi loci principle (Mohan, 2011).

Genotyping methods

The limitations associated with phenotypic subtyping methods along with the rapid growth of molecular techniques have led to the development of a wide range of molecular subtyping methods (Fitzgerald et al., 2008a). While phenotypic traits form the basis of phenotyping, genes responsible for the production of those phenotypic characters form the foundation for genotyping (Mohan, 2011). Molecular methods have become widely applied to subtype *Campylobacter* jejuni since they provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeablility, and discriminatory power, when compared with phenotypic typing methods (Eberle and Kiess, 2012; Wassenaar and Newell, 2000; Wiedmann, 2002). These may be divided into two broad categories: macro-restriction mediated analyses based on separation of restriction enzyme digested nucleotide sequences, and polymerase chain

reaction (PCR) based assays (Mohan, 2011).

Pulse field gel electrophoresis (PGFE), also known as field alteration gel electrophoresis (FAGE) or macro-restriction profiling PFGE, has emerged as one of the best molecular approaches to analyze bacterial pathogens, including *Campylobacter* (Ahmed et al., 2012; Eberle and Kiess, 2012). The PFGE is considered the 'gold standard' for epidemiological investigations due to its enormous discriminatory power (Sails et al., 2003a). Although the interpretation of PFGE data is difficult, rendering this technique unsuitable as a tool for routine use during outbreak investigation (Sails et al., 2003a), it has been extensively used in genetic and epidemiological investigations of *C. jejuni* and *C. coli* (Ahmed et al., 2012; Mohan, 2011).

The polymerase chain reaction (PCR) has certainly revolutionized molecular epidemiological studies thanks to its versatility and ability to detect the presence or absence of an organism in any sample by detecting a specific gene unique to the particular organism of interest (Mohan, 2011). Several variations of the original PCR technique have been developed and are applied for detecting *Campylobacter* spp., including reverse-transcriptase PCR, multiplex PCR, and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Notably, multiplex PCR assays, which are used for simultaneous differentiation of *Campylobacter* spp., have replaced monoplex PCR assays which were widely used for detection and differential diagnosis of *Campylobacter* spp. in the past (Asakura et al., 2008; Yamazaki-Matsune et al., 2007). These techniques are easy to reproduce, highly discriminatory, available in most laboratories and though may be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp. (Eberle and Kiess, 2012).

Apart from PCR being used as a diagnostic tool itself, most of the genotyping techniques are PCR based since it is simple, rapid, and cost effective (Asakura et al., 2008). Random amplified polymorphic DNA analysis (RAPD) and amplified length polymorphism (AFLP) are two PCR-based methods used for *Campylobacter* genotyping which provide good discriminatory power, although, due to certain limitations, these are not used successfully as a routine genotyping tool (Mohan, 2011). Ribotyping is an rRNA approach for the identification of bacterial isolates, which though has a high level of typeablility for *Campylobacter* spp., its low number of ribosomal genes gives it poor discriminatory power (Eberle and Kiess, 2012). Flagellin typing, using restriction fragment length polymorphism (RFLP), is another technique used for typing of *Campylobacter* species. Although flagellin gene typing is quick and can have high discriminatory power, it is recommended that it should not be the sole technique used in epidemiological grouping of isolates, and, therefore, it is often used in combination with other typing techniques mostly MLST (Dingle et al., 2005; Eberle and Kiess, 2012; Mohan, 2011).

DNA sequencing of one or more selected bacterial genes represents another genetic subtyping method (Wiedmann, 2002), which is becoming increasingly automated and, consequently, is a reasonable alternative method for genotyping bacterial isolates (Wassenaar and Newell, 2000). Multilocus sequence typing (MLST) is a genotypic typing method that was first developed in 1991 based on the well-established principles of MLEE (Maiden et al., 1998). This technique differs from MLEE in that it assigns alleles directly by DNA sequencing of 7 to 11 housekeeping genes rather than indirectly through the electrophoretic mobility of their gene product (Eberle and Kiess, 2012). An important component of the MLST approach is the availability of databases (e.g. PubMLST) for use by public health and research communities, where the sequence data can be compared. In turn, researchers can submit the results of their findings to these databases (Maiden, 2006).

MLST is currently the leading molecular typing method for *Campylobacter* (Ross, 2009). An increasingly used in epidemiological studies MLST system specific for the characterization *C. jejuni* strains was developed by Dingle et al. (2001), while an extended MLST method able to characterize not only *C. jejuni* but also *C. coli, C. lari,* and *C. upsaliensis,* was designed by Miller et al. (2005). The advantages of using MLST include high discriminatory power, reproducibility, ease

of interpretation and transferability of information among laboratories (Dingle et al., 2001; Wassenaar and Newell, 2000), however, it is a complex and expensive technique to perform (Ahmed et al., 2012; Djordjevic et al., 2007; Lévesque et al., 2008). Moreover, recent work has shown that the seven loci used may be insufficient to provide an accurate picture of gene content in all areas of the *C. jejuni* genome (Taboada et al., 2008). MLST is also unable to distinguish closely related strains in short-term outbreak investigations, and additional methods like fla typing may be required in order to obtain sufficient resolution (Sails, et al., 2003b).

Comparative genomics, namely the analysis and comparison of two or more genomes, has also served to underscore some of the new challenges in bacterial genotyping and phylogenetic analysis (Ross, 2009). Comparative genomic fingerprinting (CGF) is a novel method of comparative genomics-based bacterial characterization which is based on the concept that differential carriage of accessory genes can be used to generate unique genomic fingerprints for genotyping purposes (Ross, 2009). Taboada et al. (2012) developed and validated a rapid and high-resolution 40-gene comparative genomic fingerprinting method for *C. jejuni* (CFG-40). The results obtained with this method suggest that it has a higher discriminatory power than MLST at both the level of clonal complex and sequence type; while, it is also rapid, low cost, and easily deployable for routine epidemiologic surveillance and outbreak investigations (Clark et al., 2012; Taboada et al., 2012). It was shown that CGF and MLST are highly concordant, and that isolates with identical MLST profiles are comprised of isolates with distinct but highly similar CGF profiles.

CONCLUSIONS

Campylobacteriosis has become the leading foodborne disease worldwide and therefore a lot of effort is being done to achieve early diagnosis of human cases using a wide variety of direct and indirect detection methods along with specific identification tests, while epidemiological investigations of campylobacteriosis outbreaks using the innovative and constantly developing typing and subtyping systems available are increasingly conducted, providing information to recognize outbreaks of infection and match cases with potential vehicles of infection. No sole technique is perfect, thus the development of a novel typing method that combines efficiency with efficacy, while overcomes the shortcomings of currently used methods, is considered crucial

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Advanced diagnosis of vibriosis among some marine fishes in lake Temsah, Egypt

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ABSTRACT. Vibriosis is a serious disease affecting marine water fishes. The present study was applied on 320 marine fishes of four equal different species (*Sparus auratus, Mugil cephalus, Mugil seheli and Tilapia zillii*) each 80. Fishes were collected seasonally and randomly from Lake Temsah in Ismailia governorate from September 2015 to August 2016. The signs and lesions of severe septicemia, hemorrhages and ulcerations were observed among the examined fishes. Isolated bacteria were observed as yellow and green pigmented colonies on TCBS media, and as creamy colored colonies on TSA media with 2.5% NaCl concentration. The causative agent was identified as vibrio sp. Concerning gene expression, most isolates were molecularly identified using the pvsA gene primers giving a product size of 338-bp size and 348-bp for V. alginolyticus and V. parahemolyticus respectively. The highest prevalence of vibriosis was recorded in T. zillii (63.75%), M.seheli (37.5%) then M.cephalus and Sparus auratus (28.75%) while the total prevalence was (39.69%). The highest seasonal prevalence was recorded in summer (81.25%) followed by spring (35%) then autumn (23.75%) and winter (18.75%). The highest prevalence of organ specifity was in liver, kidneys then spleen and gills. The histopathological studies showed activation of melanomacrophage centers, degeneration, necrosis and congestion in liver, kidney, spleen, and gills.
Keywords: Marine fishes, Vibriosis, pvsA gene, Histopathological exam.

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INTRODUCTION

Egypt depends on fish as one of the essential sources of the national profit, stimulating local market economies and essential source of foreign exchange. Furthermore, marine waters are the main sources for water needed for mariculture and luckily, Egypt has more than one marine resources such as the Red and Mediterranean Sea (Sadek, 2000).

The universal trend of commercial aquaculture is towards condensation of culture practice in the target to increase productivity per unit area (Elgendy et al., 2015 and Kolkovski, et al. 2011).

One of the common genera in aquatic habitat, particularly in marine, is genus vibrio, several species of it are pathogenic for freshwater especially where organic loads are high (Alicia et al., 2005).

One of the most frequent diseases occurring in marine aquaculture, is vibriosis caused by many vibrio species (Alcaide, 2003). It is a serious epizootic disease affecting both wild and farmed marine fish species worldwide, and has become a limiting factor for the development of intensive aquaculture industry. Several factors have been proposed to influence the survival, persistence and ability of vibrios to cause infection (Lipp et al., 2002).

The present study was conducted to investigate the prevalence of different vibrio spp. among some marine fishes in relation to clinical picture, conventional and advanced diagnosis using pvsA gene along with histopathological examination.

MATERIALS AND METHODS

1- Fishes:

A total number of 320 marine fishes of four equal various species, average body weights 100±5 for Sparus auratus and Mugil cephalus and 50±5 for Mugil seheli and Tilapia zilli, each were 80 were represented as (Sparus auratus, Mugil cephalus, Mugil seheli and Tilapia zillii). They were collected seasonally and randomly. They were examined freshly from Lake Temsah in Ismailia governorate, Egypt from September 2015 to August 2016 and subjected to full clinical, postmortem and bacteriological examinations.

2- Bacterial examination:

a-Isolation:

The bacterial isolates were taken from ulcers, gills, liver, kidneys and spleen of naturally alive and freshly dead infected Sparus auratus, Mugil cephalus, M. seheli and Tilapia zillii. They were streaked on TCBS agar and incubated at 27ºC ±1ºC for 24-48 hr. Suspected colonies were subcultured on TSA (2.5% NaCl) and subjected to microscopic and biochemical analysis according to Farmer et al., (1992).

b-Phenotypic Identification:

b.1) Morphological and biochemical examination:

The pure colonies were identified morphologically according to Cruickshank et al., (1982) and biochemically according to Thompson et al., (2004). They were identified using the traditional biochemical tests only on Gram negative, catalase positive and Oxidase positive. These tests were accomplished by API20E strips (BioMerieux, France).

b.2) Molecular characterization of Vibrio alginolyticus & Vibrio parahemolyticus using pvsA gene:

b.2.1) Genomic DNA Extraction:

Genomic DNA was extracted from V. alginolyticus and V. parahemolyticus strains using boiling technique according to (Devi et al. 2009).

b.2.2) Oligonucleotide primers:

Four primer sequences, two sense (F) and two antisense (R), were used for identification of both strains. The primers were designed from the published pvsA gene sequences (accession no. DQ201184.2 and AB082123.1) for V.alginolyticus and V.parahemolyticus strains respectively using the web-based software Primer3Plus (Untergasser et al., 2007). Primers sequences and characteristics are shown in Table 1.

b.2.3) PCR and agarose gel electrophoresis:

The PCR reaction mixture was contained 5.5µl genomic DNA,12.5µl PCR master mix,1µl of forward primer,1µl of reverse primer in a total volume of 25µl. Reaction cycles were performed using TC-25/H thermal cycler , the cycle conditions as a follow: pre- denaturation at 94ºc for 3 min then followed by 35 cycles of denaturation at 94ºc for 30

Table 1: *V. alginolyticus* and *V. parahemolyticus* pvsA gene Primers sequence and characteristics.

Primer name	Reference gene for location	Location (np)	Sequence	Tm(ºC)	Gc%	Product size
VA 1F	DQ201184.2	10391-10410	CAG TAA CCG CCT TAC CGT GT	60.1	55	338 bp
VA 1R		10709-10728	CAC TCC AGC GTG TCG ACT TA	60	55	
VP 1F	AB082123.1	721-740	TTC CCT GTC GTA GCA AAA CC	60.1	50	348 bp
VP 1R		1049-1068	AAA TTC GCT GTG GCA AAC TC	60.3	45	

sec, annealing at 60ºc for 30 sec; and extension at 72ºc for 3 min. in order to confirm the amplification of the target sequences the PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide, the resulting fragments were visualized by UV transillumination (Slime lineTM series).

3-Experimental infection:

A total of 60 apparently healthy *Tilapia zillii*, weighting 30 ± 5g were selected for detection of the pathogenicity of the most common bacterial isolates. Fish were classified into duplicate three groups each contained 10. The inocula processed for isolated bacteria as I/P and I/M injections were prepared according to Austin & Austin, (2007). Fish were noticed daily for 14 days. one group was consistently inoculated I/P and the other group was inoculated I/M with the bacterial suspension of *Vibrio alginolyticus* in a dose of 0.2 ml of (3 X 107 CFU). The control groups were injected I/P and I/M with 0.2 ml of sterile tryptic soya broth.

4-Histopathological examination:

Specimens were collected freshly from liver, kidney, spleen and gills of naturally infected fishes. Fixed in 10% phosphate buffered formalin and processed by traditional method, sectioned at 5micron thickness. They were stained with H&E stain then examined microscopically according to Roberts, (2001).

RESULTS

A-Clinical examination of naturally infected fishes:

The clinical examination of naturally infected fishes was recorded in Plate (1).

B-Results of bacteriological examinations:

The colonies were creamy color on tryptic soya medium with a range of (2-3 mm in diameter) while on TCBS medium, they were yellow and green colored colonies with yellow pigmentation. Also, sensitive to vibriostate O/129(150 µg). Isolates were Gram- negative, comma or rod shaped scattered in arrangement and motile. The result of the conventional and commercial systems using API20E for biochemical tests declared the results in (Table 2).

Plate 1: A. Naturally infected *M.cephalus* showing severe hemorrhage on the abdomen, pectoral and tail fin. B. Severe hemorrhagic gills & kidneys and enlarged spleen. C. Naturally infected *M.seheli* showing severe external hemorrhage on external body and detached scales. D. pale gills and liver in advanced case. E. Naturally infected *S.auratus* showing hemorrhagic gills, liver and kidneys. F. Hemorrhage under the dorsal fin. G. Naturally infected *T.zillii* showing severe abdominal distention and turbid eye. H. Hemorrhagic kidneys and enlarged spleen.

C-Molecular characterization of V.alginolyticus & V.parahemolyticus using pvsA gene:

Fig 1 shows the amplification of 338 bp PCR amplicon representing pvsA gene in *V.alginolyticus* strain using VA1F and VA1R primer pair. While, Fig

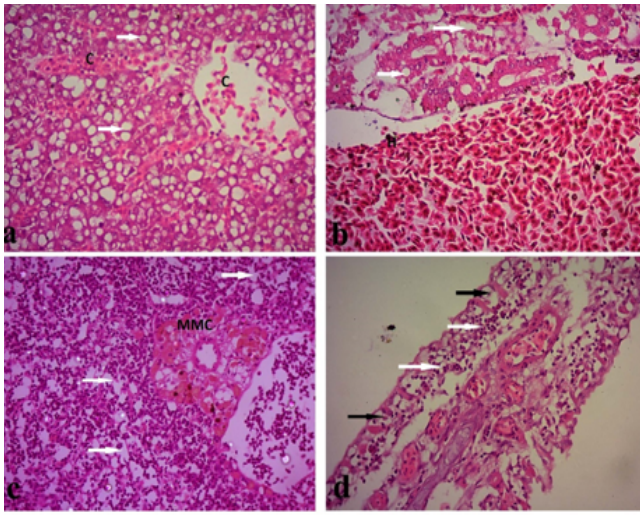

Plate 2: *Histopathological* changes induced by *Vibrio spp*. in *Tilapia zilli* fish., H&E. X 400 (a) Liver showing congestion of central vein & hepatic sinusoids and presence of distinct fat globules (arrow). (b) kidney showing massive hemorrhage and degenerative changes of renal tubules epithelium (arrow). (C) Spleen showing hyperplasia of melano-macrophage centers, congestion of blood vessels & sinusoids and depletion of white pulps (arrows). (d) Gills showing focal congestion, leukocytic infiltration (white arrows), atrophy of secondary lamellae and vacuolar degeneration of lamellar epithelium (black arrows).

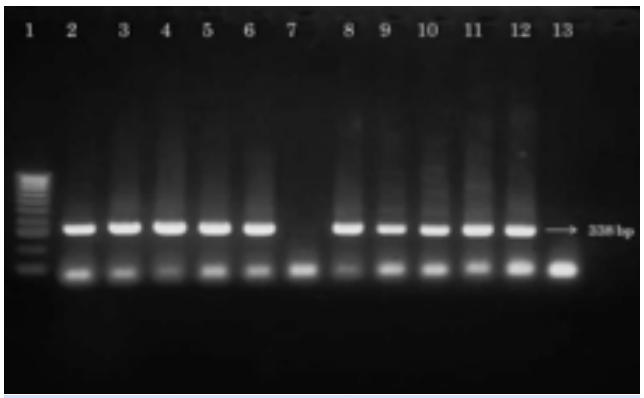

Figure 1. Showing 338- bp PCR amplicon representing pvsA gene in V.alginolyticus, Lane 1: 100 bpDNA ladder.

2 shows the amplification of 348 bpPCR amplicon representing pvsA gene in V.parahemolyticus strain using VP1F and VP1R primer pair.

D-Prevalence of vibriosis among naturally infected examined marine fishes:

1) Total and Seasonal prevalence of vibriosis among naturally infected marine fishes: results are summarized in figures 3 and 4

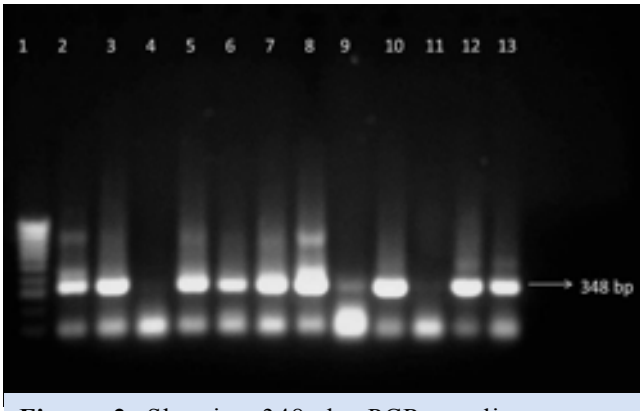

Figure 2. Showing 348- bp PCR amplicon representing pvsA gene in *V.parahemolyticus*, Lane 1: 100 bpDNA ladder.

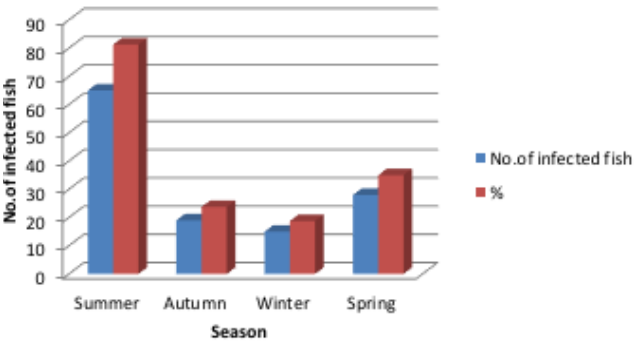

Figure 3. Showing the total prevalence of Vibriosis among the examined marine fishes.

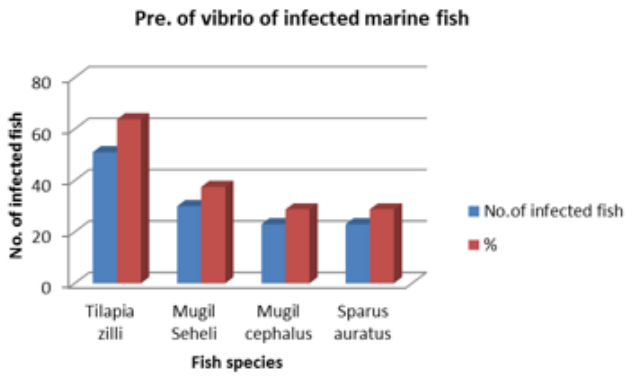

Figure 4. Showing the prevalence of Vibriosis in infected marine fishes.

2) Prevalence of vibrio spp. isolates in different organs of examined fishes:

Results are shown in figure 5.

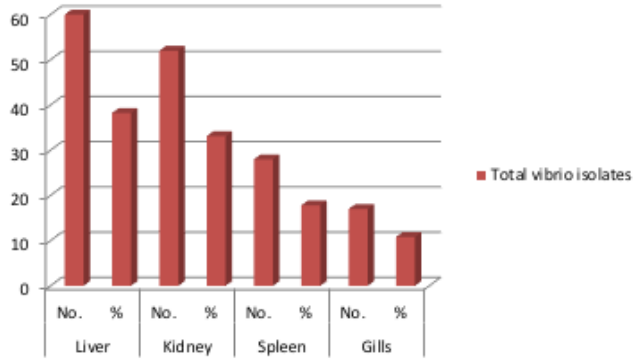

Figure 5. Showing numbers and percentages of vibrio isolates from (liver, kidney, spleen & gills) of examined fish.

E-Experimental infection:

Results of histopathological examination

Results are expressed in (plates 2,3,4,5) for *Tilapia zilli*, *Mugil cephalus, M.seheli* and *Sparus auratus* respectively.

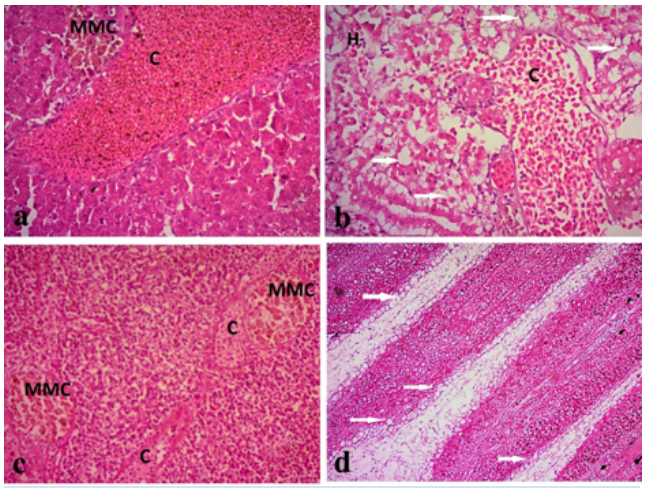

Plate 3. Histopathological changes induced by Vibrio spp. in *M. cephalus* fish .H&E X400. (a) liver showing severe congestion of blood vessels and activation of melano-macrophage centers. (b) kidney showing severe congestion and extensive degeneration (arrows) & necrosis of renal tubules along with focal hemorrhage. (c) Spleen of showing congestion of blood vessels, activation of melano-macrophage centers and mild depletion of white pulp. (d) Gills showing congestion of blood vessel, hyperplasia of primary & secondary lamellae and severe mucinous degeneration (arrows).

Discussion:

Regarding to clinical signs, it was revealed that darkness of external body surfaces & hemorrhages at abdominal and anal regions, swollen abdomen, turbid eyes, ulcer and detached scales in *T. zillii.* Also, there was redness all over the external body surface and

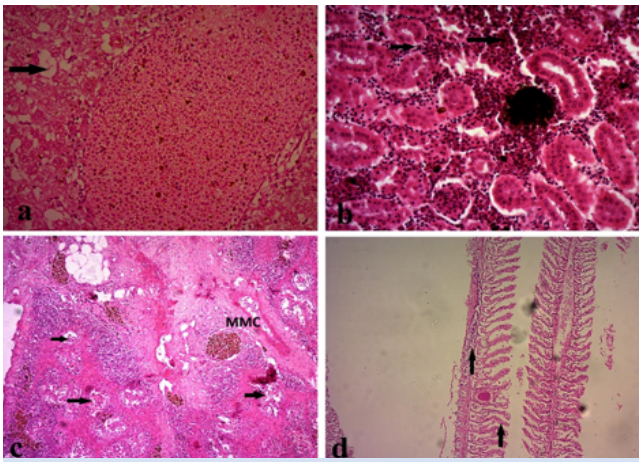

Plate 4. Histopathological changes induced by *Vibrio* spp. in *M.seheli* fish. H&E X400. (a) liver showing severe congestion, vacuolar degeneration of hepatocytes and mild focal necrosis also observed (arrows). (b) kidney showing focal inter-tubular hemorrhage admixed with leukocytic infiltration (white arrows) and mild degeneration of renal tubules with focal hyperplasia of melano-macrophage centers. (c) Spleen showing depletion and necrosis of lymphoid follicles (arrows) and hyperplasia of melano-macrophage centers (MMC). (d) Gills showing severe congestion , loss of secondary lamellae and mild leukocytic infiltration (arrows).

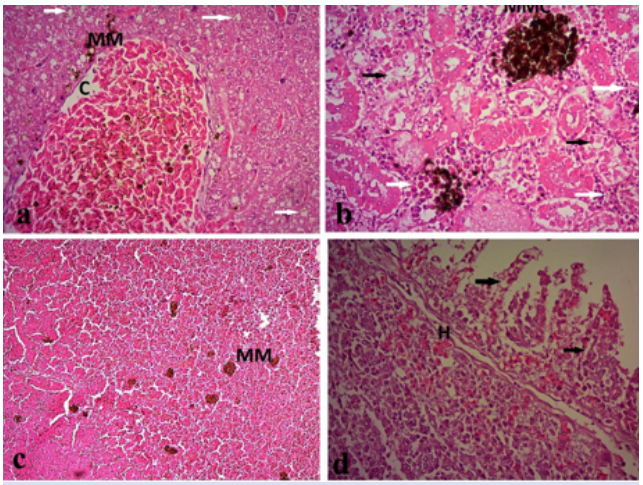

Plate 5. Histopathological changes induced by *Vibrio* spp. in *Sparus auratus* fish. H&E X 400(a) Liver showing severe congestion of blood vessels and mild to moderate vacuolation of hepatocytes (arrows). (b) kidney showing severe vacuolar degeneration of renal tubules (black arrows), focal leukocytic infiltration (white arrows) and hyperplasia of melano-macrophage centers. (c) Spleen showing hyperplasia of melano-macrophage centers, congestion of sinusoids and mild degeneration of white pulp (d) Gills showing focal hemorrhage, leukocytic infiltration, degeneration and necrosis of secondary lamellae (arrows).

pectoral fin, hemorrhage with detached scales in some areas in *M.seheli.* Other cases showing external hemorrhages and gill hemorrhages in *M.cephalus*. Besides, there was hemorrahage under the dorsal fin, turbid eye, hemorrhagic gills and pale gills in severe cases. These results agree with the findings of Alicia et al., (2005) and Eissa et al., (2013). These signs may be attributed to the adhesion ability which is an important factor in bacterial pathogenicity since it precedes penetration of the microorganisms in the host tissues promoted by the production of toxins according to Lee, (1995).

Regarding the postmortem lesions in cases suffering from vibriosis, we noticed that in naturally infected *S. auratus,* there was slight congestion in gills and liver while in severe cases; there was pale liver and gills. Some cases showing congestion in the body cavity and kidneys with yellow serous mucus in the intestines. In naturally infected *T.zillii* there was pale liver & gills, and in other cases revealed congested and hemorrhagic kidneys and inflamed intestines filled with mucus and enlarged gall bladder. Otherwise, in naturally infected *M.seheli* there was pale liver with hemorrhagic spots and pale gills. Some cases in M.cephalus showed hemorrhagic gills and enlarged spleen. These results agreed nearly with Golomazou et al., (2006), Robert et al., (2012) and Eissa et al., (2013).This may be attributed to the manner of infection in fish consist of three main steps: (i) the bacterium penetrates the host tissues by chemotactic motility; (ii) bacterium deploys iron-sequestering systems within the host tissues the, e.g., siderophores, to "steal" iron from the host; and (iii) the bacterium damages the fish by means of extracellular products, e.g., hemolysins and proteases according to Larsen and Boesen ,(2001).

The master factor that permits vibrios to survive and can cause infection within their host is their iron-sequestering systems (Tolmasky et al., 1988). These systems center upon the production of iron-scavenging compounds which known as siderophores and the consequent transport of the ferric-siderophore complex back into the cell cytosol (Crosa, 1997and Actis et al., 1999).

So, according to researchers Anzaldi and Skaar, (2010) and Kustusch et al., (2011) who found that there are iron-scavenging compounds siderophores known as vibrioferrin in *vibrio* species, these siderophores are the base of iron-sequestering systems in these bacteria helping them to uptake of iron from tissues of the hosts to their cytsol. So, it could be investigated that bacteria used iron into infected fishes and that lead to decrease their concentration in the different organs.

In this study, the isolated bacteria were gram –ve motile, fermentative catalase and oxidase positive, VP, H2S, URE –ve and there were yellow and green colonies and code number on API20E strips was 0146125 & 4047125 for *V. alginolyticus* and 401604 for *V. parahemolyticus* while Buller, (2004) described *V. anguillarum* isolated from fish as Gram-negative, motile and fermentative in O/F test. Isolates were positive for oxidase, catalase and Voges proskauer. On the other hand, it was negative in respect to H2S and urease tests. It grows well on TCBS producing yellow colonies.

According to Tomotaka et al., (2003), several genes are expected to be involved in the biosynthesis and transport of vibrioferrin have been identified in the surrounding genomic regions of the pvuA gene. One of these genes called pvsA gene which constitute an operon that is expressed under iron-limiting conditions. In this study concerning gene expression most isolates were molecularly identified using the pvsA gene primers giving a product size of 338-bp size and 348-bp for *V. alginolyticus* and V. *parahemolyticus* respectively.

In the present study, the seasonal prevalence of vibriosis was found that the highest was recorded in summer (81.25%) followed by spring (35%) then autumn (23.75%) and winter (18.75%). The seasonal prevalence among examined fishes revealed that; in *T. zillii* was (95%) in summer followed by spring (75%) then autumn (50%) and winter (40%), In *M. seheli* was (80%) in summer followed by spring (30%) then (25%) autumn and winter (15%), In S. auratus was (70%) in summer followed by spring (20%) then (10%) autumn and winter (15%), in *M.cephalus* was (80%) in summer followed by 20,10 and 5% for spring ,autumn and winter respectively. These results nearly agreed with Eissa et al., (2013) who found that the highest seasonal prevalence

was recorded in summer followed by spring then autumn and winter. Also, it was found that seasonal prevalence of *Vibrio alginolyticus* in *S. auratus* and *T. zillii* high in summer followed by in spring then autumn and at the last the winter. Also, these result in agreement with Lee et al., (2006) and Ming et al., (2011) who all confirmed that the temperature is the main predisposing factor of vibriosis and help in its development.

Owing to prevalence of vibriosis in the examined fishes, the highest prevalence was recorded in *T.zillii* (63.75%) followed by *M.seheli* (37.5%) then *S.auratus* and *M.cephalus* (28.75%) .Which nearly similar to Enany et al., (2011) who reported that *V.alginolyticus* was isolated from *M.capito* with percentage 37.5%. These results agree with Wafeek et al., (2007) who isolated *V.alginolyticus* from Grey mullet fish *(M.cephalus)* collected from Sharm El-Sheikh with high percent (39%). This may be attributed to the high immune response in S.auratus and M.cephalus rather than T.zilli.

Regarding to total prevalence, it was found in examined species (39.69%) which is slightly higher than which recorded by Eissa et al., (2013) who revealed that the total prevalence of vibriosis among naturally infected marine fishes (36%).The result is lower than that obtained by Adebayo-Tayo et al., (2011) who recorded (44.2%) of examined seafood samples obtained from Oron creek infected with vibrio spp. Also, it was lower than which obtained by Balebona et al., (1998) who reported (67.8%) in three fish farms with intensive culture of gilt-head seabream, S.aurata L. in southwestern Spain infected with vibrios. That attributed to number of fish, age, site of collection and water temperature.

Regarding to prevalence of vibriosis in different organs of examined fishes, It was shown that the highest prevalence of vibriosis in liver (38.22%) followed by kidneys (33.12%) then spleen (17.83%) and gills (10.8%) which are similar to Eissa et al., (2013) who recorded the same arrangement in *V.alginolyticus*. These results can be explained that, the liver and kidneys are the common target organs of infection. Similarly to El-Bassiony, (2001) who reported that liver was the highest organ (4 isolates) followed with kidney and spleen about (3 isolates) And not agreed with Wafeek et al., (2007) who approved that the distribution of V.alginolyticus in different organs was higher in mouth, gills, liver, kidneys and spleen.

Concerning to challenge test, the mortality rate increased in *T. zillii* that mostly high in the first three days post inoculation in the two inoculation methods (I/P ,I/M) then decreased continuously reached the total mortality rate 80% and 70% respectively The re-isolation and identification of inoculated bacteria from the challenged *Tilapia* proved that, it was corresponded to the same tested strain for challenge the infected fish revealed the same clinical and postmortem changes nearly as Vibriosis. Infected fish revealed ulcers, hemorrhages all over the body and dorsal fin. Besides, pale liver and gills, enlarged gall bladder and congested kidneys which agreed with Sakata and Hattori (1988) who found that experimental moribund tilapia revealed signs of a hemorrhagic septicemia as the same as the natural disease. This proved that the injected *V. alginolyticus* strain was mostly virulent.

Concerning the histopathological results induced by vibriosis in examined fishes, severe congestion, hemorrhages and degenerative changes were observed in most types of fish and this could be attributed to the severity of isolated strains. Our results come in agreement with Roberts, (2001a), El-Bassiony, (2001) and Stephens et al., (2006).

CONCLUSION

1-Seasonal prevalence of vibrio spp. was highest in summer followed by spring, autumn and winter.

2-PCR yielded amplification for V.alginolyticus and V.parahemolyticus strains isolated from some marine fish representing pvsA gene and is considered as an important tool for diagnosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effects of diet supplementation with different level of Celmanax® in rainbow trout *(Oncorhyncus mykiss)*

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ABSTRACT. The aim of this study was to evaluate the effect of a prebiotic (Celmanax®) containing yeast cell wall with mannan oligosaccharides on the haematological and serum biochemical parameters in rainbow trout. Three levels of prebiotic (0, 0.1, 0.5 and 1 %) were mixed into pellets. Fish (19.08±1.45 g) were fed a supplemented commercial diet for 60 days. Blood samples were colected from the onset and on days 30 and 60 of the trial to measure the haematological and serum biochemical parameters in rainbow trout. The results showed significant differences in haemoglobin, mean corpuscular volume,mean corpuscular haemoglobin concentration, white blood cells and neutrophil count between control and all test groups ($p<0.05$). The highest and the lowest white blood cells and neutrophil count (on day 60) were observed in the 0.1 %, prebiotic-received and control groups, respectively. Also, the result showed significant differences in Alkaline phosphatase enzymes, serum glutamic oxaloacetic transaminase, Serum glutamic-pyruvic transaminase, between the test and control groups($p<0.05$) while non-significant elevation of blood urea nitrogen, creatinine and total protein levels was found in the Celmanax®-received groups ($p>0.05$). These results suggest that the Celmanax® supplementation enhances white blood cells and neutrophil count, and changes some biochemical parameters in rainbow trout.

Keywords: Celmanax®; Rainbow trout (*Oncorhynchus mykiss*); Haematological; Biochemical parameters; Mannan-oligosaccharides

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INTRODUCTION

Worldwide interest in prebiotics has increased in animals as well as in rainbow trout. Various sectors of the aquaculture industry would benefit if cultured organisms were conferred with improved growth performance, feed efficiency, disease resistance, modulation of the gut micro biota and enhanced immune responses (Ringø et al., 2010). Since then the most common prebiotics used in fish are inulin, fructooligosaccharides (FOS), oligofructose, mannanoligosaccharides (MOS), trans-galactooligosaccharides (TOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMO) and various commercial products containing multiple prebiotic combinations. Although the potential of prebiotics may have interesting applications in aquaculture in order to improve growth performance, survival, feed conversion, digestibility, gastrointestinal (GI) enzyme activities, immune functions and the presence of beneficial gut bacteria as well as the suppression of potentially pathogenic bacteria. In addition, several papers have investigated the effect of prebiotics on GI morphology (Pryor et al. 2003; Genç et al. 2007; Dimitroglou et al. 2009; 2010; Ringø et al. 2010; Sweetman et al. 2010; Dimitroglou et al. 2011a; 2011b; 2011c). Salmonids such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are among the most well documented fish species in respect to prebiotic applications. Indeed, MOS, GOS, FOS, inulin, and commercial products containing multiple prebiotic combinations have been investigated in studies on Atlantic salmon, brook trout (*Salvelinus fontinalis*), rainbow trout and arctic charr (*Salvelinus alpinus*) (Merrifield et al., 2010 and Ringø et al., 2010). In a recent study, Rehulka et al., (2011) reported results from a 105-day experiment, where the effect of dietary scFOS on rainbow trout (initial weight 240±34.9 g) growth and plasma biochemical parameters was determined. Inclusion of scFOS at 1 g kg−1 did not significantly affect Special Grow Rate (SGR), Feed Conversion Ratio (FCR) and survival. The results of the biochemical parameters indicated significant differences in creatinine, Na+ and alkaline phosphatase (ALP, involved in intestinal mucosal defence).

The prebiotic, Celmanax™ (Vi-COR®, Mason City, IA, USA), consists of a non-living formulation of yeast cell walls, mannan oligosaccharide (MOS) and yeast metabolites (*Saccharomyces cerevisiae*). Haematological parameters changes would be sign of fish physiological responses against environmental stresses e.g. such as pH alteration, salinity of water pollution or bacterial infections (Zorriehzahra, 2010). Thus important internal organs such as kidney, spleen, liver and pancreas that have important duty in fish physiology must be affected by infectious pathogens to change the haematological items in response to the invading pathogens. In many cases of fish infectious diseases diagnosis could be assisted by haematological study. Several studies have demonstrated that prebiotic of MOS can improve the growth parameters, survival, haematological and biochemical parameters, gut morphology and modulate the intestinal microbiota in various aquatic species, including sea bass (*Dicentrarchus labrax*) (Torrecillas et al., 2007), rainbow trout (Staykov et al., 2007; Dimitroglou et al., 2009), atlantic salmon (Grisdale-Helland et al., 2008); (Andrews et al., 2009), sea bream (*Sparus aurata*) (Gultepe et al., 2011), Japanese flounder (*Paralichthys olivaceus)* (Ye et al., 2011). Haematological parameters are repeatedly used as diagnostic and effective tools to assess the health status of fish (Thrall et al., 2012). Knowledge of haematological parameters of Salmonidae family are frequently used as an essential diagnostic tool to assess the growth and health condition. Haematological parameters are closely response of the animal to the environment and indication that the effect of diet on the haematological characteristics.

Rainbow trout (*Oncorhynchus mykiss*) is a fish belonging to the family of Salmonidae and native to the cold water rivers and lakes of the Pacific coasts of North America and Asia; the habitat and food of rainbow trout determine both their actual colour and shape (Roozbahani, et al., 2009). Since 1874 *O. mykiss* has been introduced to all continents except Antarctica, for recreational angling and aquaculture purposes, because it is a resistant fish, easy to spawn, fast growing and capable of occupying many different habitats because it can tolerate a wide range of environmental and production conditions better than other trout species (Parisi et al., 2014).

Today, nearly all rainbow trout on the European Union market come from aquaculture. Most of the EU supply of trout is locally produced. Currently the largest producers worldwide are Chile, Norway, Iran and Turkey. In 2016, a total of one million tons of seafood was grown for human consumption in Iran. The amount was 82000 tons more than in 2015. However, out of this, about 140,000 tons were rainbow trout. The rest consisted of sturgeon and caviar, shrimp and other fishes.

Rainbow trout is one of the most commercially important species grown in Iran and all around the world. Hence, the objective of the experiments was to determine the effects of Celmanax® prebiotic on haematological and some biochemical parameters of cultured rainbow trout.

MATERIALS AND METHODS

Experimental design

Rainbow trout (19.08±1 g) were purchased from a commercial fish farm in Urmia, west Azerbaijan province, Iran. Acclimatization to the laboratory condition was performed for 10 days in 1000 L tanks using aerated free-flowing well water with the following characteristics: temperature (13.5 ± 1ºC); pH (7.5); dissolved oxygen (8 ± 0.2 mg/L); natural photoperiod (10 h light/14 h dark); flow rate (1.25 l/s). Fish were fed three times daily with commercial fish feed (40% protein), 3% of average initial body weight per day.

Diet preparation and feeding trial

Commercial basal diet (21 Baiza, Shiraz, Iran) was used in this study; three experimental diets, commercial diet supplemented with 0.1% (T1), 0.5% (T2) and 1% (T3) Prebiotic. After spraying the different level of Celmanax® on commercial feed, pellets were dried at room temperature for 2 h and then the diets were stored at 4°C until use. Fish were randomly divided into 4 groups (in triplicate) of 50 animals per tank and were fed for a 60-day period. On days 0, 30, 60 a sample of three individuals per tank (nine per treatment) was taken to measure haematological and biochemical parameters.

Haematological parameters

Fish were anesthetized with 200 mg/L clove oil, then blood was collected from cardinal vein using heparin coated syringe and transferred into sterile tubes. The blood was allowed to clot at room temperature for 1 h and stored in a refrigerator overnight. The clot was then centrifuged at 1500 × g for 5 min. Then the serum was collected and stored in sterile eppendorf tubes at -20ºC until use for biochemical assays. Also, blood collected by caudal vein puncture in heparinized syringes. Haematocrit values (Ht) were determined by centrifuging fresh blood in heparinised glass capillary tubes for 5 min. Haemoglobin (Hb) level was determined colorimetrically by measuring the formation of cyanomethaemoglobin using a commercial kit (Pars Azmoon, Tehran, Iran). Red blood cells (RBCs) and white blood cells (WBCs) were counted under a light microscope using a Neubauer haemocytometer after dilution with phosphate buffered-saline (PBS). Deferential leukocyte counts (neutrophil, lymphocyte, eosinophil and monocyte) were determined using blood smears under a light microscope. Cells were identified on the basis of morphology and cell ultra-structure as documented in previous fish leukocyte studies (Jalali et al., 2009).

Biochemical parameters

In this study alkaline phosphatase (ALP), Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic-pyruvic transaminase (SGPT), Blood urea nitrogen (BUN), Creatinine and total protean were assayed in collected serum samples. An automatic blood enzyme analyser (Hitachi 704) was used for the following determinations: Alkaline phosphatase, Serum glutamic oxaloacetic transaminase, Serum glutamic-pyruvic transaminase, Blood Urea Nitrogen, Creatinine and total protean serum. The apparatus is based upon dry chemical technology and colorimetric reaction. Kits obtained from PrasAzmoon®, Iran, were used for the determination of all parameters.

Statistical analysis

The data (Mean ±SD) were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test to compare the means between individual treatments with SPSS (Version 21; SPSS Inc.,) at $p < 0.05$ level.

Table 1. Haematological parameters in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment at start of the study.

Hematological parameters	Control	T1	T2	T3
Haematocrit (%)	32.15± 1.2 [a]	32.17±1.2 [a]	32.8±1.1 [a]	33.1±1.3 [a]
Hemoglobin (g/dl)	6.24±0.1 [a]	5.11±0.1 [a]	5.86±0.1 [a]	6.60±0.1 [a]
RBC (10^6 cell/mm3)	1.06± 0.1 [a]	1.00±0.18 [a]	1.009±0.12 [a]	1.00±0.18 [a]
MCV (Fl)	303±5 [a]	301±5 [a]	305±5 [a]	305±5 [a]
MCH (pg)	58.67±0.5 [a]	58.77± 0.5 [a]	58.64 ± 0.5[a]	58.55± 0.5 [a]
MCHC (g/dl)	17.20±0.5 [a]	16.90±0.5 [a]	18.15±0.5 [a]	18.50±0.5 [a]
WBC (10^3 cell/mm3)	13.72±0.2 [a]	14.02±0.1 [a]	13.85±0.2 [a]	14.10±0.2 [a]
Lymphocyte (%)	89.30±1.01 [a]	90.32±1.21 [a]	89.8±1.2 [a]	89.16±1.4 [a]
Neutrophil	6.78±1.1 [a]	6.2±1.7 [a]	7.1±1.1 [a]	7.08±1.0 [a]
Eosinophil	3.21±0.1 [a]	2.89±0.2 [a]	3.2±0.6 [a]	3.4±0.3 [a]
Monocyte	0.58±0.1 [a]	0.5±0.1 [a]	0.54±0.1 [a]	0.49±0.1 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

RESULTS

The effect of dietary Celmanax® on rainbow trout blood profiles is presented in Tables 1-3. Red blood cell count and haematological parameters such as haematocrit, Eosinophil and Neutrophil count did not significantly differ between treatments and control (p>0.05). The highest haematological parameters value at day 60 of study were recorded HB (7.23±0.1 g/dL), MCHE (21.61±0.9 g/dL), WBC (14.80±0.3 cell/mm3), Lymphocyte (92.41±0.1%), Neutrophil (7.24±1.7%), Monocyte (1.7±0.1%) in treatment 0.1% Celmanax® diet and RBC (1.47±0.08×106

Table 2. Haematological parameters in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment at day's 30 of the study.

Hematological parameters	Control	T1	T2	T3
Haematocrit (%)	31.81±2 [a]	30.53±2 [a]	29.36±2 [a]	29.18±1 [a]
Hemoglobin (g/dl)	5.34±0.1 [a]	5.51±0.1 [a]	5.47±0.1 [b]	5.38±0.1 [c]
RBC (10^6 cell/mm3)	0.895±0.1 [a]	0.862±0.01 [a]	0.847±0.02 [a]	0.88±0.01 [a]
MCV (Fl)	332.65±3 [a]	329.42±5 [a]	328.05±5 [a]	316.32±5 [b]
MCH (pg)	61.89±0.5 [a]	59.86±0.5 [d]	60.53 ± 0.5 [c]	61.32± 0.5 [b]
MCHC (g/dl)	23.46±0.5 [a]	17.13±0.5 [c]	16.21±0.5 [d]	18.37±0.5 [b]
WBC (10^3 cell/mm3)	14.07±0.01[c]	14.82±0.01 [a]	14.32±0.01 [b]	14.41±0.2 [b]
Lymphocyte (%)	91.50±0.1[a]	89.54±0.1 [a]	89.97±0.1 [a]	90.12±0.3 [a]
Neutrophil	6.33±1.0 [c]	9.93±1.7 [a]	9.55±0.5 [b]	9.37±0.1 [b]
Eosinophil	0.5±0.1 [a]	0.6±0.2 [a]	0.4±0.1[a]	0.4±0.1 [a]
Monocyte	0.6±0.1 [a]	0.45±0.1 [a]	0.48±0.1 [a]	0.51±0.1 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 3. Haematological parameters in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment at day's 60 of the study.

Haematocrit (%)	32.18±1.3 [a]	32.52±1.2 [a]	33.56±2.1 [a]	33.28±2.3 [a]
Hemoglobin (g/dl)	6.27±0.1 [d]	7.23±0.1 [b]	6.74±0.1 [a]	7.07±0.1 [c]
RBC (10^6 cell/mm3)	1.21±0.1 [a]	1.29±0.11 [a]	1.36±0.01 [a]	1.47±0.08 [a]
MCV (Fl)	228.24±5 [d]	238.28±5 [b]	236.33±5 [c]	241.05±5 [a]
MCH (pg)	51.86±0.5 [a]	50.11± 0.5 [c]	49.59 ± 0.5 [d]	51.08±0.5 [b]
MCHC (g/dl)	19.13±0.5 [d]	21.61±0.9 [a]	20.11±0.5 [b]	19.45±0.5 [c]
WBC (10^3 cell/mm3)	11.70±0.2 [c]	14.80±0.3 [a]	13.02±0.2 [b]	12.90±0.2 [b]
Hematological parameters	Control	T1	T2	T3
Lymphocyte (%)	91.24±0.1 [a]	92.41±0.1 [a]	91.73±1.2 [a]	91.86±2.4 [a]
Neutrophil	6.26±1 [c]	7.24±1.7 [a]	7.1±1.1 [b]	6.38±1.0 [b]
Eosinophil	0.64±0.1 [a]	0.59±0.2 [a]	0.52±0.23 [a]	0.40±0.1 [a]
Monocyte	1.1±0.1 [a]	1.7±0.1 [a]	1.2±0.1 [a]	1.5±0.1 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet)

cell/mm3), PCV (2.3±33.28%), MCV (241.05±5 Fl) were in treatment 1% Celmanax® diet. However, the lowest value of MCH (49.59±0.5pg) which were in treatment 0.5% Celmanax® diet lower the respective control in all the treatment groups. In day 60 of study, Total white blood cell (WBC) was significantly different (P<0.05) between the prebiotic-received groups compared to control. Similar trends was recorded in the value of neutrophil. The values of Monocyte in all the treatment up than control and were raised above the control with highest value recorded at in treatment revised 0.1% Celmanax® diet. Eosinophil value in the fish varied widely with both increase and decrease relative to the control value. The results in day 60 showed significant differences in Haemoglobin, MCV, MCH, MCHC, white blood cells and neutrophil count between the control and treatment groups (P<0.05). Evaluation of alkaline phosphatase enzyme showed significantly (p<0.05) decreased between control (1002±1) and all treatment groups (T1:664± 10, T2: 645± 5 and T3: 928±3). In day 60 of study, the highest decrease of alkaline phosphatase was seen in treatment 0.5% Celmanax® diet (T2: 645± 5) (table 4). The results also showed that addition of Celmanax® on to the diet in day 60, significantly (p<0.05) increased the SGOT enzyme in two treatments (T2:399±10 and

Table 4. Alkaline Phosphatase level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	1255±1 [a]	1221±5 [a]	1296±1 [a]	1252±3 [a]
30	1658±5 [a]	1608± 10 [b]	1306±5 [c]	711±10 [d]
60	1002±1 [a]	664± 10 [c]	645± 5 [d]	928±3 [b]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 5. SGOT level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	807±5 [a]	795±5 [a]	818±1 [a]	790±3 [a]
30	597±5 [d]	724± 5 [c]	751±5 [b]	892±5 [a]
60	464±1 [b]	399± 10 [a]	635± 5 [d]	484±3 [c]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

T3:484±3) and SGPT enzyme significant increase in two treatments (T1: 21±5 and T3:15±4), however, there was significant difference among the prebiotic-received groups in different days. Also in day 60, SGOT level was significantly ($p<0.05$) decreased between 0.1% Celmanax® diet treatments (399±10) and control (464±1) (tables 5-6). However, a non-significant ($p>0.05$) change among the Blood urea nitrogen (BUN), Creatinine and total protean levels was found in groups prebiotic-received compared to control ($p>0.05$) (table 7-9).

Table 6. SGPT level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	22±5 [a]	20±3 [a]	18±1 [a]	19±2 [a]
30	13±1 [b]	21±4 [a]	13±1 [b]	12±1 [b]
60	7±3 [c]	21±5 [a]	10±1 [c]	15±4 [b]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 7. Blood urea nitrogen level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	3±1 [a]	4±1 [a]	4±1 [a]	3±1 [a]
30	4±1 [a]	3±1 [a]	2±1 [a]	3±1 [a]
60	3±1 [a]	3±1 [a]	3±2 [a]	2±1 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 8. Creatinine level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	0.75±0.05 [a]	0.45±0.05 [a]	0.30±0.05 [a]	0.35±0.05 [a]
30	0.34±0.02 [a]	0.30±0.01 [a]	0.31±0.01 [a]	0.41±0.01 [a]
60	0.20±0.01 [a]	0.30±0.01 [a]	0.24±0.02 [a]	0.25±0.03 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 9. The serum Total protein level in rainbow trout fed diets with Celmanax® prebiotic. Each value (X ± SD) is the average performance of nine fish per treatment during the study.

Day	Control	T1	T2	T3
0	3.32±0.05 [a]	3.85±0.05 [a]	3.21±0.1 [a]	3.45±0.1 [a]
30	3.35±0.05 [a]	3.98±0.15 [a]	3.56±0.1 [a]	3.44±0.1 [a]
60	3.19±0.2 [a]	3.25±0.3 [a]	3.66±0.5 [a]	3.29±0.1 [a]

*The same superscript alphabets in the same row indicate a non-significant different at P<0.05. (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

DISCUSSION

Hematologic evaluation of fish is not routinely used in establishing the diagnosis of fish diseases, but it can be useful in the detection of diseases and different materials in the food affecting the cellular components of blood. Certain diseases of fish result in anemia, leukopenia, leukocytosis, thrombocytopenia, and other abnormal changes of the blood cells. Evaluation of the hemogram also may be useful in following the progress of the disease or the response to therapy.

Routine assay methods for the biochemical evaluation of mammalian blood appear to be useful for fish blood; however, interpretation of the results can be difficult. Many endogenous (species, age, nutritional status, gender, reproductive status) and exogenous factors (environmental conditions, population density and method of capture) influence the plasma biochemistry results of fish (Thrall et al., 2012).

In the present study, SGPT, SGOT and ALP levels were affected by Celmanax® Commercial product ($p<0.05$). The liver tissue of teleosts appears to be rich in aspartate aminotransferase (AST) and possible alanine aminotransferase (ALT). Therefore, plasma activity of these enzymes may elevate with severe hepatocellular disease in some piscine species. High activities of AST and creatine kinase (CK) also occur in muscle of fish; therefore, elevated plasma activities of these enzymes will increase following muscle injury or strenuous muscle activity associated with capture and restraint (Thrall et al., 2012).

In recent years, less studies have been done on the efficacy of probiotics on blood parameters and enzymes in aquaculture. In 2007, Welker et al. reported that 0.2% dietary MOS had no effect on

the WBC, RBC, Hct, Hb and total serum protein of channel catfish *(Ictalurus punctatus*). Sado et al. (2008) also reported that 0.2-1% MOS had no effect on tilapia (*Oreochromis niloticus*) RBC, WBC, Hb, Hct, MCV, MCH, MCH, MCHC and plasma total protein. In the prebiotics research, Ahmdifar et al. (2011) and Hoseinifar et al. (2011) who reported that prebiotic inulin and oligofructose had no effects on SGPT, SGOT and ALP enzymes in beluga *(Huso huso)* serum, also Amani Denji et al., (2015) report no significant differences in serum enzymes activity (SGPT, SGOT and ALP), cholesterol, triglyceride and total protein among treatment use 1, 2.5 and 4 gr /kg MOS prebiotic in rainbow trout.

Some studies on effect of prebiotics, in contrast to the above results and showed the effect of prebiotic on blood parameters and enzymes. In this study, the diet supplementation with Celmanax® in all treatments too changed Alkaline Phosphatase level in rainbow trout (table 4). Alkaline phosphatase activity occurs in multiple tissues including bone and intestine, and increased plasma AP activity results not from leakage of the enzyme but from increased cellular production. Metabolism of minerals in the liver and bone have a direct effect on Alkaline Phosphatase level in serum. The increase in the Alkaline Phosphatase could be due to the rapid growth phase and the production of collagen in the cartilage before bone formation. Ninety percent of the secretion of the alkaline phosphatase enzyme is related to the liver and bones, and small amount to the intestines. The Alkaline phosphatase plays a role in the metabolism of minerals, distribution of carrier proteins and the activity of DNA and RNA polymerases. In hepatic damage, the activity of alkaline phosphatase and gamma-glutamyl transferase are increased in blood.Isoenzymes of alkaline phosphatase (L-ALP, CL-ALP) increase in bone growth and various diseases, too (Bargerand MacNeill, 2015). In this study, probably bone growth could be the reason of alkaline phosphatase increase in treatment Fed with Celmanax®.

Bone mass of an adult is dependent on supply as well as bioavailability of calcium. Even though, a lot of studies have been carried out on calcium metabolism using rats, results depicted that prebiotics play a role in escalating the bioavailability of calcium (Wong et al., 1988).

On day 60 of the study, there was a significant difference in secretion of SGPT enzyme between the groups fed with the 0.1% of Celmanax® between control (P=0.001) and the group fed with 1% of Celmanax® between control (P=0.01). Considering the significant increase in the SGPT in fish need for additional histopathological observations is required. Blood urea nitrogen, Creatinine and total protean of the control and experimental groups were not significantly affected by addition of Celmanax® to the diet (P>0.05) in all treatment groups and the prebiotics were not affected to these enzymes.

In other study Anguiano et al, (2012) showed effects of four prebiotics (fructo-oligosaccharide, Bio-MOS, transgalacto-oligosaccharide and GroBiotic-A) on digestive enzymes and intestinal morphology in juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*) and red drum (*Sciaenops ocellatus*). The results of this study showed no significant changes in the enzyme activities were detected at week 8 in both species (Anguiano et al. 2012). In 2015, Amani Denji et al. reported the effect of dietary MOS (Active MOS®; Biorigin, LencoisPaulista, Sa¨o Paulo, Brazil) supplementation on rainbow trout (1, 2.5 and 4 gr /kg MOS). Result of this study showed ALT, AST and ALP activities did not significantly (p>0.05) differ between treatments. In 2015, Gültepe et al., showed effects of prebiotic MOS on the histology and biochemical blood parameters of the gilthead sea bream (*Sparus aurata*). MOS additives to feed did not have a significant effect on AST, ALT and ALP. Adel et al., (2016), reported Activities of lysozyme and alkaline phosphatase in *Huso huso* skin mucus were significantly enhanced in Grobiotic®-A fed group, particularly at an inclusion level of 1% and higher (2% group compared to the control).

The results of the present study showed that dietary containing Celmanax® had effects on Hb, MCV, MCH, MCHC and WBC and Neutrophil counts (neutrophilia). Neutrophils are a critical component in the first line of defense against invading pathogens. Multi-receptor recognition of PAMPs and DAMPs define intruding pathogens, resulting in the activation of cellular antimicrobial responses designed to kill infiltrating pathogens. Antimicrobial responses are tailored to the type and

location of the pathogen, and can be divided into two main categories: intracellular and extracellular. Intracellular defense mechanisms are designed to provide protection against pathogens found within membrane-enclosed structures. These defenses are not limited to killing pathogens that have been internalized through phagocytosis, but also to provide protection against pathogens actively hiding from humoral immune defenses. Neutrophils are armed with an extensive antimicrobial arsenal designed to limit the dissemination of a broad range of pathogens. Interestingly, many of the antimicrobial mechanisms present in teleost neutrophils are utilized both as intracellular and extracellular defences (Havixbeck et al, 2015).

According to the results of Marzouket al. (2008), a positive effect represented by significant increase in differential leukocytes count, these could be attributed to the fact that, the probiotics used increased the blood parameter values because of haematopoietic stimulation. However, WBC levels, particularly neutrophil, were elevated in fish fed dietary containing Celmanax® (Tables 2,3). This was similar to the results of Andrews et al., (2009), who observed a significant improvement in WBC, RBC and Hb in rohu *(Labeo rohita)* fed MOS supplemented diet in comparison with those fed on the control diet.

In other study, Talpur et al., (2014) showed effects of commercially available probiotic and prebiotics on growth performances, haematological and immune response and disease resistance in *Channa striata* fingerlings against the pathogenic bacteria *Aeromonas hydrophila*. Dietary probiotic and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against *A.hydrophila* in Snakehead (*Channa striata*) fingerlings. Fish were fed six different diets up to 12 weeks containing single dosage of *Lacto acidophilus* at 1 g kg-1 feed (1010CFU), yeast 1%, β-glucan 0.1%, Galactooligosacharide (GOS) 1% and Mannan oligosaccharide (MOS) 0.2 % and control without any supplement. Dietary probiotic and prebiotics improved haematology parameters. There was a significant improvement in red blood corpuscles (RBCs), white blood corpuscles (WBCs), pack cell volume (PCV), haemoglobin (Hb%) concentration, erythrocyte sedimentation rate (ESR) and serum protein content in treated groups over the control (Talpur et al., 2014).

A similar study was conducted on the effect of Mannan oligosaccharide supplementation in carps by Momeni-Moghaddam et al., in 2015. Momeni-Moghaddam et al., results showed administration of MOS at 0.05-0.20% improves FCR, modulates intestinal microbiota and at 0.20% elevates the humoral immune response of common carp by increasing the alternative complement activity, lysozyme activity and serum total immunoglobulin (Momeni-Moghaddam et al., 2015).

CONCLUSIONS

The administration of probiotics varies from oral/water routine to feed additives, of which the latter is commonly used in aquaculture. Probiotic applications can be either mono or multiple strains, or even in combination with prebiotic, immune stimulants such as symbiotic and symbiotic, and in live or dead forms. Accordingly, data in this study showed that addition of Celmanax® (0.1% diet) to the rainbow trout diet could order better Hb, MCV, MCH, MCHC, white blood cell and neutrophil rate with values statistically higher than those from the control. In conclusion, the results of this study show that the addition of Celmanax® in died could increase the immunity performances, betterment alkaline phosphatase level and supposition improve phagocytosis (neutrophilia) and health management in aquaculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Comparative behaviour studies of growing dairy and beef bulls from two different breeds

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ABSTRACT. This study aimed at comparing the behaviour of growing bulls from two different breeds – Lithuanian black and white (dairy cattle breed, n=22) and Aubrac (beef cattle breed, n=17). The hypothesis tested in this study was that breed differences would result in differences in the behaviour of the bulls and that a clear understanding of such differences could lead to improved husbandry to meet their needs. Animals were raised in insulated barn in pens with deep litter, both breeds under similar conditions (animals were 11 to 14 month old, each group in separate 100 m^2 pen). Aubrac bulls spent more time in standing, ruminating, drinking and aggressive behaviour and less time in eating than the Lithuanian black and white bulls. There were no significant differences in lying and moving behaviour. These differences possibly related to different breed of animals, and should inform decision making about the management of the two breeds studied.

Keywords: behaviour; bulls; Aubrac cattle; Lithuanian Black-and-White cattle.

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INTRODUCTION

In order to ensure proper keeping conditions for cattle, to maintain and improve their welfare and the safety of animals and personnel there is a need for some breed-specific information about animal behaviour. Different breeds of cattle can be characterized by different behaviour and temperament attributes (Grandin, 1989; Piovezan et al., 2013). In particular, there are some behavioural differences between dairy and beef cattle (Murphey et al., 1980). There is tendency that cattle of larger size, originating from colder environments, are calmer compared to smaller sized beef cattle breeds found in warmer regions (Grandin and Deesing, 2013). Aubracs are almost the same size as Lithuanian black and white cattle (Piedrafita et al., 2003; Jukna et al., 2006). The Aubrac breed originated from France, a warmer region compared to Lithuania. The Lithuanian Black and White (BW) was developed in the early 20th century by crossing local cattle with Ostfriesian, Swedish Black and White, Holstein-Friesian, and other breeds (Baltrėnaitė et al., 2003). This breed perfectly represents average local European dairy cattle.

Beef husbandry is a newly developing branch of cattle husbandry in Lithuania. At the beginning of 2001, there were only 19 farms keeping 346 purebred beef cows, of which one-third (123) were of the Aubrac breed (Jukna, 2001). By 2015, there were more than 4,000 purebred Aubrac cattle, of which there were about 1,400 heifers, 1,400 cows and 1,200 bulls. Aubracs account for 14 % of all beef cattle raised in Lithuania (Lithuanian centre of agricultural information and rural business, 2016). In Lithuania, there is a tendency to have mixed cattle breed farms, where dairy farmers support the financial viability of their businesses with beef cattle as an additional production source. In such cases, all male offspring of dairy cows are kept in the same conditions as hybrid or purebred beef bull calves. This situation raises some questions about the welfare and behavioural needs of such different animals. Therefore, the objective of this study was to compare the behaviour of growing bulls from two different breeds – Lithuanian black and white (dairy cattle breed) and Aubrac (beef cattle breed) kept under similar conditions. It was hypothesized that breed differences would result in differences in the behaviour of the bulls and that a clear understanding of such differences could lead to improved husbandry to meet their needs.

MATERIAL AND METHODS

The experiment was conducted on the cattle farm of the Institute of Agriculture (Lithuania) in Dotnuva (55° 23’ 42.37”, 23° 51’ 29.4”). Animals were managed according to the Lithuanian regulations regarding the use of animals in scientific experiments. Two groups of 11 to 14 month old bulls, one group of the Aubrac (n=17) and the other group of the Lithuanian Black-and-White breed (n=22) were observed. There was no statistically significant difference between the two groups with respect to age. At the start of the experiment mean weights of the Aubrac and Lithuanian Black-and-White bulls were 372.7±8.9 kg and 296.9±5.7 kg respectively. The bulls of the Aubrac and Lithuanian Black-and-White breeds were housed in the same insulated barn, which consisted of straw-bedded pens in four rows. Animals were kept in group pens with concrete flooring (one pen per group, 100 m^2/pen) on deep straw litter. The lying area was cleaned out once at the end of the finishing period. All bulls were fed the same diets based on grass silage and barley-based concentrates. Pen measurements (10 m × 10 m) and feed trough widths (10 m) were the same for both groups. Aubrac bulls had a space allowance of 5.88 m^2 per bull while black and white bulls had 4.55 m^2 per bull. This was slightly higher than the current minimum recommended space allowance for beef cattle in this type of housing in Lithuania (4 m^2 per animal, Lithuanian Ministry of Agriculture, 2009). Bearing in mind that the Aubracs were heavier, space allowance per kg of live weight was 0.0158 m^2 for Aubracs and 0.0153 m^2 for black and white bulls, so that conditions similar for both groups.

The behaviour of the bulls was observed for a period of 24 hours (00:00–24:00 hours) in June. Average air temperature indoors was 16^{0}C, and relative humidity was 75%. The bulls were observed using instantaneous sampling (Dawkins, 2007) with a 5-min sampling interval. At each

Table 1. Description of postures and activities recorded during instantaneous sampling observations.

Behavior	Description
Lying total	Lying in any position.
Feeding at the feeding trough	Eating and masticating silage or barley at the feeding trough.
Drinking	Drinking water from the bowl.
Standing ruminating	Chewing cud in a standing position.
Lying ruminating	Chewing cud in a lying position.
Moving	Walking without interaction with feed or bedding
Standing idling	Standing without any apparent activity.
Lying resting	Lying without any apparent activity in any position with trunk in contact with ground. Includes sleeping.
Aggressive	Interactions between at least two animals, involving head to head contacts (Bouissou et al.2001)

sampling point each bull was scanned and the posture and activity of the bull were registered according to the classification method and descriptors presented in Table 1 (Tuomisto et al., 2015).

The results are presented in absolute and relative terms (min, %). Behavioral data obtained from scan sampling were expressed in minutes assuming that each behaviour persisted for the entire 5-minute scan interval. Values were expressed as means ± SEM. Mean comparisons were made using the Student two-sample unequal variance (heteroscedastic) t-test with a two-tailed distribution. A t-test's effect size was calculated as Cohen's d coefficient. Results were considered statistically significant at $P < 0.05$.

RESULTS

Frequencies of the observed behaviours are presented in Table 2.

There were no significant differences between the two groups of animals as regards their frequencies of lying and moving behaviours. Aubrac bulls spent more time standing, ruminating, drinking and less

Fig 1: Lying behaviour of the Lithuanian Black-and-White and Aubrac bulls in the course of 24 hours.

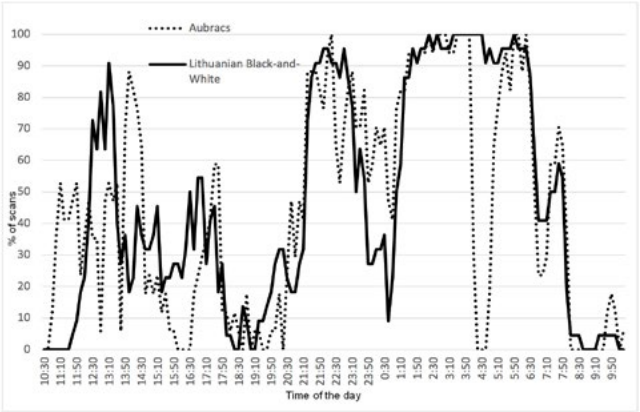

Figure 1. Lying behaviour of the Lithuanian Black-and-White and Aubrac bulls in the course of

time eating than the Lithuanian black and white bulls. Despite the fact that there were no significant differences in lying behaviour frequencies, Lithuanian black-and white bulls spent more time lying with less lying bouts compared with the Aubrac bulls (Table 2). The bulls of the Aubrac breed were distinguishable by more frequently expressed aggressive behaviour in the group. The mean time per animal spent in aggressive

Table 2. Behaviors of Aubrac and Lithuanian black-and-white bulls kept indoors.

Measures	Lying		Moving	Standing			
	Resting	Ruminating		Idling	Ruminating	Eating	Drinking
			Aubracs n=17				
minutes per animal per day	321	332	247	154	295	24	
	652		68	720			
No. of lying bouts per day	9						
% of scans during 24 h period	22.3 ±1.8	23.0 ±1.6	17.2 ±1.5*	10.7 ±1.1*	20.5 ±2.0*	1.6 ±0.3**	
	45.3 ±3.0		4.7 ±0.9	50.0 ±2.8			
			Lithuanian black and white n=22				
minutes per animal per day	294	383	180	113	390	10	
	677		71	692			
No. of lying bouts per day	6						
% of scans during 24 h period	20.4 ±1.8	26.6 ±1.7	12.5 ±1.1	7.9 ±0.8	27.1 ±2.3	0.7 ±0.2	
	47.0 ±3.0		5.0 ±0.8	48.0 ±2.8			

* $P < 0.05$; ** $P < 0.01$

behaviours accounted for 4.2% of the total time in 24 hours (39 encounters between two or more bulls) compared with a mean of 1.5% of time (21 encounter) for the Lithuanian black-and white bulls (data not presented in the table).

There were no statistically significant differences between the breeds regarding any of the diurnal fluctuations in behaviours analysed. Bulls of both breeds expressed a clear diurnal rhythm - during the night almost all bulls spent most time lying (Figure 1) and during the day, most time was spent performing other behaviours. During the period between 20:30 and 8:00 Aubrac bulls spent a little less time lying (70.4 % of observations, Figure 1) than the Lithuanian Black-and-White bulls (73.1 % of observations, p=0.58, Figure 1). The same tendency was observed during the daytime e.g. during the period between 8:10 and 20:20 Aubrac bulls spent a little less time lying (21.5 % of observations, Figure 1) than the Lithuanian Black-and-White bulls (22.3 % of observations, p=0.84, Figure 1). During the period between 8:00 and 12:20, most Lithuanian Black-and-White bulls (average 63 %) spent their time eating (Figure 2). Later, between 12:30 and 13:20 most of these bulls (average 65 %) started to ruminate in lying position. Meanwhile Aubrac bulls started to eat at 8:20 and most animals (average 72 %) spent their time eating until 9:50 (Figure 2). Later eating activity was combined with ruminating and idling behaviours in an irregular manner. The most significant differences in lying behaviour was observed in the period between 14:40 and 16:30, when there were only 0-23.5 % (average 10.3 %) of

Fig 2: Feeding behaviour of the Lithuanian Black-and-White and Aubrac bulls in the course of 24 hours.

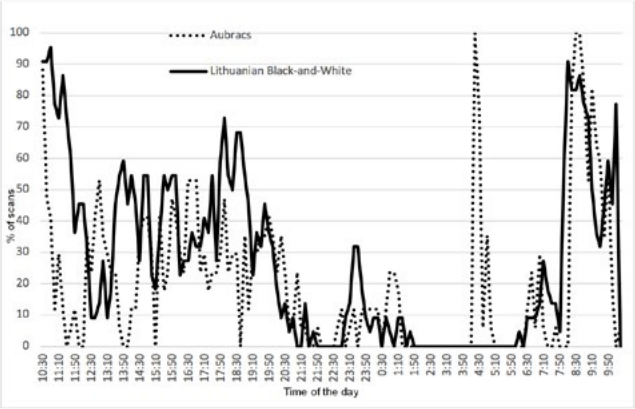

Table 3. Synchronization of behaviors among individuals of each breed, observed at different percentage categories of scans showing the same posture (standing/moving or lying).

Animals	Amount of scans, %			
	70	***80***	***90***	***95***
	% of animals showing the same posture			
Lithuanian BW bulls	74.3	60.4	48.6	38.9*
Aubrac bulls	75.7	60.4	39.6	25.0

* $P < 0.05$

Aubrac bulls lying compared to 18-50 % (average 30.7 %, P < 0.01) of the Lithuanian Black-and-White bulls (Figure 1). Effect size (expressed as Cohen's d coefficient) for statistically significant differences is 0.3.

Behaviour synchronization of the observed animals was not statistically different at the 70 and 80 % scan levels (Table 3). At the 90 and 95 % thresholds, behaviour synchronization of Aubrac bulls was lower compared to that of the Black-and-White bulls. This means that 90% (and more) and 95 % (and more) of Aubrac bulls were observed showing the same posture (lying or not lying) at 39.6 and 25 % of all observations, while 48.6 and 38.9 % of observations of Lithuanian Black-and-White bulls showed behavioural synchronization from 90 and 95% of the scans made.

DISCUSSION

In this study, the behaviour of the bulls from two different breeds (Lithuanian black and white and Aubrac) in insulated barn in pens with deep litter were compared.

The study showed a clear diurnal rhythm of cattle behaviour, which was similar to that found by Robért et al. (2011). They found that most cattle lay between 20:00 and 04:00. In this study, it was found that most of the Aubrac bulls spent their time lying in the periods between 21:00 - 04:00 and 05:00 - 07:00. Most Lithuanian black and white bulls spent their time lying in the periods between 21:00 – 24:00 and 01:00 – 07:00 that is during the dark period of the day. There were clear periods of activity caused by the morning (08:00) and evening (18:00) feeding periods of the animals. Therefore, in our study, we observed a clear overall drop in activity during the nighttime (with some differences between breeds) and a rise in activity during the light period of the day and during the feeding period of the animals.

It was found that total lying times of the Lithuanian black and white and Aubrac bulls were 652 and 677 min per day respectively (p>0.05). Absmanner et al. (2009) found that the total lying time of 450-600 kg Simmentals kept in group pens on straw bedding was 780 min. Hickey et al. (2014) found that total lying time of 335 kg Charolaise heifers kept on slatted floors was 768 min. Cook (2008) found that mean lying time of 208 dairy cows in cubicles was 660 min. (168-1056 min.) which was the closest finding for lying timings compared to the findings in the present study.

In the present study, total eating times of Lithuanian black and white and Aubrac bulls were 295 and 390 min per day respectively (p<0.05). Hickey et al. (2014) reported that the total eating time of Charolais heifers was 318 min. Cook (2008) found that the mean eating time of dairy cows in cubicles was 264 min (84-468 min). Gottardo et al. (2003) found that Simmentals (around 321 kg) spent 50% of their time lying, while we report a little less, 45.3% (Aubracs) and 47.0% (Lithuanian BW). Eating and rumination times in this study were similar to those found by Gottardo et al. (2003), 20-30% and between 30 and 40% respectively. Aubrac bulls had nine lying bouts per day (table 2), while the Lithuanian black and white bulls had a mean of six lying bouts per day. These findings are similar to those reported by Hickey et al. (2014), who found that Charolais heifers kept on slatted floors had 6.7 lying bouts and 9.2 lying bouts per day at pasture. Ipema et al. (2010) reported that lying of dairy cows during day was divided over seven bouts, varying in length between 11 and 137 min, and that cows kept in a straw yard had more lying bouts than cows kept in a cubicle barn with slatted floor. One can assume that Aubrac bulls having more lying bouts were more active compared to the black and white bulls. The

Aubrac lying bouts were shorter and interrupted by frequent periods of activity. This may have been related to the higher number of agonistic events among the Aubrac bulls, which could have disturbed the animals from lying.

There were differences in observed aggressive behaviour between the breeds; the Aubrac bulls attacked each other more often compared to the black and white bulls. The mean number of encounters between bulls from both groups was similar to that recorded for 321 kg weight Simmental bulls (Gottardo et al., 2003) – from 10 to 40 times per day. Brscic et al. (2007) reported that the number of agonistic behaviours (fights and mounts) between 508 kg weighing finishing French crossbred bulls was 34.6 per day in hot conditions, 30.5 in mild and 32.3 in cold conditions.

The behaviours of a cattle group are usually determined by the diurnal rhythm (light, feeding, other regular events or farm management activities), individual characteristics or preferences of animals and synchronization of behaviour of the entire group. Cattle have been demonstrated to show synchronization of lying and standing behaviours (Stoye et al., 2012). Such synchronization is identified when cattle lie down or stand up at the same time as other members of their herd. In the present study, it was found that behaviour synchronization of both groups was similar. Most of the animals (70 % and more) showed the same behaviours (lying or standing/moving) in 75.5 and 74.3 % of all scans for Aubrac and Lithuanian Black and white cattle respectively (Table 3). At the 90 and 95 % thresholds, behaviour synchronization of Lithuanian black and white bulls was slightly greater (48.6% and 38.9%) than that of the Aubracs (36.6% and 25.0%, $P < 0.05$, Table 3). It may therefore be assumed that, compared to Lithuanian black and white cattle, some of the Aubrac bulls were more independent of the rest of their herd and were less influenced by the behaviour of other individuals. Social behaviour of cattle (including synchronization) can be influenced by the housing conditions: number of animals kept in the same pen and floor space available to them, quality of surface at the lying place and human-animal relationships (Waiblinger et al., 2001). Lying behaviour could also depend on housing and management conditions (O'Driscoll et al., 2008). Because of differences in housing and management conditions, it is difficult to compare the behaviour synchronization results among cattle that have been reported in different studies.

Stoye et al. (2012) estimated that time of the day has some importance for the synchronization of cattle behaviour. The lowest rate of synchronization was found in the middle of the day, while greater synchronization was found during the morning and evening. In this study, the same effect was observed. In the middle of the day, cattle expressed more diverse behaviours than during the morning, evening and nighttime. Behaviour synchronization, especially lying synchronization can be seen to reflect some degree of comfort and a better social environment for cattle (Phillips and Schofield, 1994). There have been attempts to use cattle behaviour synchronization as a welfare indicator (Miller and Wood-Gush, 1991; Fregonesi and Leaver, 2001; Napolitano et al., 2009) but of the multiplicity of secondary factors such as time of the day, available space, number of animals in the group, other housing conditions and management factors might limit the use of synchronization as a universal indicator for assessing welfare.

Despite fact that total lying time was not significantly different between groups, the bulls of the Aubrac breed had more lying bouts and fewer animals lay during the nighttime. Together with the lower behaviour synchronization score, this allows the assumption that Aubrac bulls might be more individual, less influenced by the behaviour of other animals in the group, than Lithuanian black and white bulls. This kind of behaviour is characteristic of less tame cattle or breeds of animals with a less docile temperament (Grandin and Deesing, 2013).

CONCLUSIONS

Overall, this behavioural study revealed only minor differences in time budgets between the two breeds of cattle. There were no differences in lying and moving between Aubrac and Lithuanian black and white bulls. The Aubrac bulls spent more time in

standing, ruminating, drinking and less time in eating than the Lithuanian black and white bulls. The bulls of the Aubrac breed were distinguished by more frequently expressed aggressive behaviour in the group. These differences resulted most probably from the different temperaments of the two breeds of cattle studied. There is evidence that Aubrac breed cattle are less socially integrated than Lithuanian Black and White cattle. This understanding should inform best practice of the management of these animals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

A Comparative Study of Seminal Plasma and Blood Serum Macro and Trace Elements in the the Breeding (October) and the Non-Breeding (April) Seasons in Merino Ram

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ABSTRACT. In this study, it was aimed to investigate the concentrations of macro and trace elements in seminal plasma and blood serum in the breeding (October) and the non-breeding (April) seasons in Merino Ram. Nineteen Merino Rams, aged 18-24 months, were involved in the study. Blood (once) and ejaculate samples (6 replicates) were taken in the breeding (October) and the non-breeding (April) seasons. Blood serum, seminal plasma and diet Calcium, Sodium, Potassium, Magnesium, Phosphorus, Sulfur, Zinc, Selenium, Chrome, Manganese, Nickel, Molybdenum and Boron concentrations were determined by ICP-AES. In blood serum, Sodium and Selenium concentrations were higher ($p<0.05$ and 0.001, respectively) in the the breeding season than in non-breeding season, whereas Potassium, Chromium and Boron concentrations were lower ($p<0.05$, 0.001 and 0.001, respectively) in the breeding season than in the non-breeding season. In seminal plasma Calcium, Sodium, Zinc and Manganese concentrations were higher ($p<0.05$, 0.001, 0.01 and 0.05, respectively) in the breeding season than in the non-breeding season, whereas Phosphorus, Chrome, Molybdenum and Boron concentrations were lower ($p<0.001$, 0.001, 0.05 and 0.001, respectively) in the breeding season than in the non-breeding season. No difference was detected regarding the other elements. The higher levels of Cr and B in the non-breeding season compared to the breeding season both in serum and seminal plasma, regardless of diet intake, suggest that these elements may play a crutial role on male fertility in Merino Ram.

Keywords: Merino ram, seminal plasma, elements, breeding season, non-breeding season.

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INTRODUCTION

Seasonal pattern including climate, humidity and daylight length are the environmental factors that influence the reproductive activity of small ruminants, causing seasonal changes in testicular size, weight, secretion, sperm production and mating activity (Folch et al., 1984; Gündoğan, 2006). Seasonal variation in sperm quality has been reported in rams of many breeds (Gündoğan, 2006; Marti et al., 2007; Mickelsen et al., 2006). The seminal plasma, which is a complex mixture of the secretions originating from the seminiferous tubules, tubuli recti, rete testis, ductuli efferentes and epididymis, serve as a nutrient medium in which maturation of the developing spermatozoa takes place (Mann, 1964). Moreover, it affects sperm morphology, motility, acrosome reaction and fertility (Mann and Lutwak-Mann, 1981). The chemical composition and the functions of seminal plasma vary among species. Cations like sodium (Na+) and potassium (K+) in the seminal plasma establish the osmotic balance (Zamiriand Khodaei, 2005) while calcium (Ca2+) is reported to stimulate steriodogenesis in leydig cells (Henricks, 1991). Trace elements are essential for the function of various enzymes and other proteins. Seminal plasma contains several trace elements that play important roles in the semen function, including sperm metabolism and capacitating, and in the acrosome reaction.

Zinc (Zn), selenium (Se), iodine (I), copper (Cu) and manganese (Mn) are trace elements that are reported to affect parameters of fertility (Leonard-Marek, 2000). Zinc is an important micronutrient for health and is known to play a major role in the semen ejaculation as well as to be a cofactor for the DNA - binding proteins. The total content of Zn in human semen is very high and is found to have critical role in the spermatogenesis (Endre et al., 1999).

Calcium is needed for stimulation of steriodogenesis in Leydig cells including sperm maturation and plays a vital role in the regulation of the motility, capacitation, hyperpolarisation and chemotaxis (Carlson et al., 2003; Suares and Ho, 2003). Magnesium (Mg) is reported to be in high concentrations in the prostate gland and may play a role in sperm motility (Edorh et al., 2003). Sodium is also reported to be present in the human seminal plasma at higher concentrations (Jeyendran and Van Der Ven, 1989). Nickel (Ni) revealed negative effects on the structure and function of testis, seminal vesicle, and prostate gland in mice (Pandey and Srivastava, 2000). Selenium is associated with albumin, glutathione peroxidase (GPx) and selenoprotein (Awadeh et al., 1998) and Se dependent enzymes protect membranes from oxidative damage, also its deficiency affected sperm quality (Leonard-Marek, 2000).

In man, the accurate contents of macro- and trace elements have been reported in detail. Abnormal levels of ionised Ca, Mg, Zn (Pandy et al., 1983) and Cu (Stanwell-Smith et al., 1983) in seminal plasma are correlated with infertility in man. On the other hand, only a few studies have been published on serum and seminal plasma macro and trace element status in animals. The breeding season and polygamy of the ram puts it in a place apart from other species that his nutrient requirements (like macro and trace elements) for semen production will be relatively high over a short breeding season (Kendall et al., 2000).

To our knowledge, there was no reported study on blood serum and seminal plasma macro and trace element status in Merino Rams comparing breeding and non-breeding seasons. Thus, the objective of the study was to investigate the concentrations of blood serum and seminal plasma macro and trace elements in the breeding (October) and the non-breeding (April) seasons in Merino Rams.

MATERIAL AND METHODS

Semen and blood samples from 19 Merino Rams (1,5-2 years of age) were used in the study. The rams, belonging to the Bahri Dağdaş International Agricultural Research Institute, Konya-Turkey (located at 37.857063 north latitude and 32.567036 east longitude), were maintained under uniform feeding, housing and lighting conditions within breeding and non-breeding seasons. Rams were fed with a ration composed of alfafla hay, concentrated feed, corn silage and dried grape, had ad libitum access to fresh water. The study was approved by Bahri Dağdaş International Agricultural Research Institude, Local Animal Research, Ethics Commette (No: 22.07.2013/2).

Ejaculates were collected from the rams using an

artificial vagina, in the breeding (October) and the non-breeding season (April) as 6 replicates (with an interval of 1 day) according to AI standard procedures (n=112 in the breeding and non-breeding seasons each, 2 samples were failed to collect). The ejaculates were cooled at 4 oC immediately after collection and centrifuged at 800 g x 10 min at 4 oC. Seminal plasma was seperated from spermatozoa for analysis in two hours. Blood samples were collected from the jugular vein at the beginning of the sperm collection, once in each season (breeding and non-breeding) . Serum and seminal plasma samples were stored at -70 oC until the analysis of macro and trace element concentrations. Starting 15 days prior to blood and sperm sampling, feed samples (n=8 for each season) were taken with an interval of 1 week.

Serum, seminal plasma and feed macro and trace element concentrations were determined with ICP-AES (Inductively Coupled Plasma Atomic Emission Spektrometer- Varian-Vista Model) using referance material European Referance Materials-LGC (ERM DA120a, Teddington, UK).

The samples of serum and seminal plasma were diluted with deionized water (total volume 1 ml) and 5 ml % 65 HNO3 + 2 ml H2O2 (Merck) was added before digestion in the microwave oven (CEM MarsXpress, Matthews, NC, USA) at 210 oC. Food samples were digested with 7 ml % 65 HNO3 + 3 ml H2O2. The flame conditions were those recommended by the instrument manufacturer for Ca, Na, K, Mg, P, S, Zn, Se, Cr, Mn, Ni, Mo, B (wavelength 393.366, 589.592, 766.490, 279.553, 213.618, 181.971, 213.856, 196.026, 267.716, 257.61, 231.604, 202.03, 249.773 nm and detection limit 0.01, 0.2, 0.5, 0.01, 10, 9, 0.3, 5, 0.5, 0.05, 0.3, 0.8 and 0.07 ppb respectively). All data was obtained using 10 second integration times based on 3 standard deviations and in general compromize conditions were used. Analyzing reference material ERM-DA120a tested the reproducibility of the method. Referance values for Se and Zn were given to be 64.1 and 658 ppb respectively in the procedure. In this study, Se and Zn levels were determined as 52 and 665 ppb, respectively.

STATISTICS

Results were expressed as the mean±S.E.M. t-test was used to determine significance between groups. Correlation between blood and seminal plasma parameters were performed with Pearson correlation. P values below 0.05 were considered to be significantly different.

RESULTS

Blood serum, seminal plasma and diet element concentrations in the breeding and non-breeding seasons were given in Table 1, 2 and 3. In blood serum, Na ($p<0,05$) and Se ($p<0,001$) levels were higher, wheras K ($p<0,05$), Cr and B levels were lower, in the breed-

Table 1: Table 1. Blood Serum Element Concentrations in Merino Rams.
During Breeding and Non-Breeding Season (ppm) (n=19) (mean±S.E.M.)

Parameter	Breeding (October)	Non-Breeding (April)	P
Ca	78.13±1.98	73.82±1.55	-
Na	1354.32±98.57	1094.76±23.57	<0.05
K	199.88±3.29	217.68±3.93	<0.05
Mg	25.74±0.57	24.87±0.57	-
P	171.74±10.31	141.50±3.28	-
S	670.78±10.38	655.73±9.57	-
Zn	0.635±0.279	0.599±0.025	-
Se	0.235±0.019	0.150±0.013	<0.001
Cr	0.041±0.007	0.069±0.005	<0.001
Mn	0.011±0.001	0.010±0.001	-
Ni	0.011±0.001	0.011±0.002	-
Mo	0.023±0.002	0.018±0.001	-
B	0.889±0.008	2.512±0.225	<0.001

Table 2: Seminal Plasma Element Concentrations in Merino Rams.
During Breeding and Non-Breeding Season (ppm) (n=112) (mean±S.E.M.).

Parameter	Breeding (October)	Non-Breeding (April)	P
Ca	86.72±2.43	79.06±2.01	<0.05
Na	1243.02±19.53	1063.82±14.87	<0.001
K	739.48±16.70	729.11±10.92	-
Mg	57.45±1.412	57.39±1.370	-
P	1798.41±41.10	2122.55±39.90	<0.001
S	295.70±7.05	238.67±6.12	-
Zn	3.812±0.363	2.706±0.0690	<0.01
Se	0.406±0.030	0.389±0.025	-
Cr	0.129±0.007	0.164±0.007	<0.001
Mn	0.063±0.008	0.041±0.028	<0.05
Ni	0.043±0.006	0.048±0.0004	-
Mo	0.022±0.002	0.026±0.002	<0.05
B	3.100±0.120	6.463±0.329	<0.001

ing season compared to the non-breeding season. In seminal plasma, Ca ($p<0,05$), Na ($p<0,001$), Zn ($p<0,01$) and Mn($p<0,05$) levels were higher, wheras P ($p<0,001$), Cr ($p<0,001$), Mo ($p<0,05$) and B ($p<0,001$) levels were lower, in the breeding season compared to the non-breeding season. No difference was determined regarding the other elements.

Table 3: Daily Intake of Elements of Rams During Breeding and Non-Breeding Seasons (ppm/ram/day) (n=8).

Parameter	Breeding (October)	Non-Breeding (April)
Ca	16667.78	12789.92
Na	1809.37	348.69
K	25815.63	26659.09
Mg	3125.45	3888.26
P	4735.54	3348.31
S	3323.08	2555.01
Zn	123.33	29.41
Se	2.065	2.789
Cr	2.239	1.879
Mn	111.70	75.26
Ni	5.409	4.035
Mo	5.227	4.178
B	40.89	53.56

There was no significant correlation ($p>0,05$) between blood serum and seminal plasma elements both in the breeding and the non-breeding seasons (Table 4 and 5).

DISCUSSION

There has been little published work on seminal plasma macro and trace element status of ram. To our knowledge, the concentrations of elements regarding the breeding and the non-breeding seasons in rams and other small ruminants have not been reported.

Seminal plasma and blood serum Zn concentrations were reported to be 488.6±76.4 µg/dl and 75.6±8.3 µg/dl respectively in Merino Ram, by Başpınar et al., (1998). Antaplı, (1990) reported blood serum Zn concentrations to be 32.5-150 µg/dl in Merino sheep and our results are in good agreement with Antaplı (1990) . Zinc is an essential element for production of sex hormones, attachment of head to tail in spermatozoa and its deficiency results in disorders of testes development and spermatogenesis (Saaranen et al., 1987; Cigankova et al., 1998). In this study, serum Zn concentrations were not significantly different (0.635±0.279 and 0.599±0.025 ppm in the breeding and the non breeding, respectively) between the seasons whereas seminal plasma Zn concentrations were higher in the breeding than in the non-breeding season (3.812±0.363 and 2.706±0.0690 ppm, $p<0.001$). The breeding season and polygamy of the ram may contribute to the fact that requirements for semen production will be relatively high in the breeding season and thus the higher Zn concentrations in seminal plasma will be required for the duration of

Table 4: Correlations (r-values) of blood serum and seminal plasma elements in the breeding season in Merino rams.

Breeding Season	Blood Ca	Blood Na	Blood K	Blood Mg	Blood P	Blood S	Blood Zn	Blood Se	Blood Cr	Blood Mn	Blood Ni	Blood Mo	Blood B
SP Ca	0,178												
SP Na		0,300											
SP K			-0,373										
SP Mg				0,097									
SP P					0,170								
SP S						0,421							
SP Zn							0,035						
SP Se								-0,001					
SP Cr									0,234				
SP Mn										-0,079			
SP Ni											-0,172		
SP Mo												-0,141	
SP B													0,263

Table 5. Correlations (r-values) of blood serum and seminal plasma elements in the non-breeding season in Merino rams.

Non-breeding season	Blood Ca	Blood Na	Blood K	Blood Mg	Blood P	Blood S	Blood Zn	Blood Se	Blood Cr	Blood Mn	Blood Ni	Blood Mo	Blood B
SP Ca	0,178												
SP Na		0,234											
SP K			-0,244										
SP Mg				-0,219									
SP P					-0,132								
SP S						0,218							
SP Zn							0,211						
SP Se								-0,197					
SP Cr									-0,292				
SP Mn										0,189			
SP Ni											-0,175		
SP Mo												0,053	
SP B													0,182

spermatogenesis (Kendall et al., 2000). The lower concentrations of Zn in the seminal plasma of infertile men compared to fertile individuals (Şeren et al., 2002) may suggest that higher concentrations of Zn in breeding season points out the importance of Zn in spermatogenezis and male fertility.

Kaneko, (2008) reported serum Ca concentrations to be 115-128 mg/L which represent higher levels from our results (78.13±1.98 and 73.82±1.55 ppm). In the present study, there was no difference in serum Ca concentrations between seasons, while seminal plasma Ca concentration was higher ($p<0.05$) in the breeding season than in the non-breeding season. The higher concentration in the breeding season may be attributed to the important role of Ca on sperm metabolism, motility, vitality, capacitation, chemotaxis and acrosome reaction (Thomas and Meizel, 1988; Carlson et al., 2003; Suarez and Ho, 2003).

Magnesium concentrations in blood serum (25.743±0.573 and 24.872±0.574 ppm, in the breeding and the non-breeding seasons, respectively) was in good agreement with (Al-Noaemi, 2007) (24.7±11.8 mg/L) in sheep. No data was found about seminal plasma Mg concentrations in ram. Magnesium is suggested to be in high concentrations in the prostate gland and is released into the semen during ejaculation. It is thought to play a role in spermatogenesis (Edorh et al., 2003). However, there was no significant difference regarding Mg concentrations between the seasons either in serum or in seminal plasma.

In adult mice, oral Mo exposure at different doses affected sperm parameters, including sperm motility, sperm count, and morphology; it increased the parameters at a moderate dose (25 mg/L), where negatively affected at high doses (≥ 100 mg/L) (Zhai et al., 2013). Marques et al., (2011) reported sheep serum Mo concentrations to be 0.31±0.16 µmol/L (0.0126-0.0470 mg/L) which are similar with our results (0.023±0.002 and 0.018±0.001 ppm in the breeding and the non-breeding seasons, respectively). No research was obtained regarding seminal plasma Mo concentrations in rams and small ruminants. In this study we could not determine any significant difference in serum and seminal plasma Mo concentrations between the seasons evaluated.

Zemanova et al., (2007) reported semen Ni concentrations to be 0.30 mg/kg in ram. In this study, the lower concentration of Ni in seminal plasma (0.043±0.006 and 0.048±0.0004 ppm in the breeding and the non-breeding season, respectively) may suggest that much of Ni in semen is contained in spermatoza. Ni deprivation in rat was reported to impair reproductive performance, decrease spermatozoa motility in epididymis (Yokoi et al., 2003). However in this study, Ni concentration did not differ between

the breeding and the non-breeding seasons neither in serum nor seminal plasma.

Blood serum Se concentrations were similar to those reported by Ghany-Hefnawya et al., (2007). Se concentration in the semen has been reported in bull and ram and Se is a component of GPx. Glutathione peroxidase in seminal plasma has been shown to have significant positive correlation with sperm number (Smith et al., 1979). Selenium is reported to be actively incorporated into the developing spermatozoa of bulls (Smith et al., 1979) and rams (Pond et al., 1983). Semen concentration of Se has been associated with sperm quality. However, serum Se did not affect sperm quality and oxidative DNA damage in human sperm (Xu et al., 2001). Pirinçi et al., (1999) reported semen Se concentrations to be 0.39-0.41 ppm in human. In this study, seminal plasma Se concentrations were 0,406 ± 0,030 and 0,389 ± 0,025 ppm in the breeding and the non-breeding season, respectively. It is reported that more than 85% of the Se is in the seminal plasma in man (Bleau et al., 1984). No data was obtained about seminal plasma Se concentrations in ram, but the agreement of seminal plasma and semen concentrations may reflect to the fact that much of the semen Se is in the seminal plasma as appeared in men. In this study, serum Se concentration was higher ($p<0.001$) in the breeding season than in the non-breeding season but, there was no difference in seminal plasma Se levels between the seasons. Considering with the diet Se levels (Higher in the non-breeding season than the breeding season, Table 3.), Se absorption seem to be lower in non-breeding season, because Se concentrations were lower in the non-breeding season compared to breeding season in blood serum, Table 1), in this study. We may suggest that, the absorption of Se seem to be at an optimal range for blood levels. Also, the role of Se in blood, regardless of seminal plasma in breeding season can be discussed. Sperm motility was reported to be maximal at semen Se levels ranging between 50 and 69 ng/ml and motility was decreased above and below this range. This result suggests an optimal range for semen Se (Bleau et al., 1984) ,regardless of serum status. However this relation is not clear.

Both serum and seminal plasma Cr concentrations were higher ($p<0.001$) in non-breeding season than in breeding season while diet Cr levels were lower in the non-breeding season than in the breeding season (Table 1, 2, and 3). The lower intake of Cr with diet and higher serum and seminal plasma concentrations in non-breeding season suggests different mechanisms may be involved in the absorbtion and distribution of this element. Chromium compounds are reported to induce oxidative stress leading to tissue damage (Stohs et al., 2001). Various studies have shown that hexavalent Cr (+6) caused testicular atrophy, reduced sperm motility and number, increased the number of abnormal sperm in adult rats and mice (Ernst, 1990; Saxena et al., 1990; Ernst and Bonde, 1992). Administration of curcumin (antioxidant) to Cr (+6)-treated rats prevented the Cr(+6)-induced spermatogenic damage, reduced testosterone level, decreased sperm count and generation of free radicals. Testicular tissue, which has high content of polyunsaturated membrane lipids, is a target for metal-induced oxidative stres (Acharya et al., 2004). However, the roles of this element in ram fertility needs further investigation.

Serum B concentrations in sheep were reported to be 1-1.5 ppm (Miyamoto et al., 2000) which is in agreement with our results. To our knowledge, seminal plasma B concentration in ram was not reported. In this study, B concentrations in seminal plasma (3.100±0.120 and 6.463±0.329 ppm, in breeding and non-breeding seasons, respectively) were much higher than in blood serum (0.889±0.008 and 2.512±0.225 ppm in breeding and non-breeding seasons, respectively) indicating the importance of this element for male reproduction. Tarasenko et al., (1972) and Krasovskil et al., (1976) reported that workers exposed to borate dusts and cadmium exhibited reduced sperm count and impaired sexual activity. High dose and long term (38 weeks) administration of boric acid in dogs caused testicular degeneration, including spermatogenic failure and atrophy of the seminiferous epithelium (Weir and Fisher, 1972). Short- and long-term oral exposures to boric acid or borax in laboratory animals have demonstrated that the male reproductive tract is target of boron toxicity. Testicular lesions have been reported in rats administered oral boric acid or borax (Weir and Fisher, 1972; Truhaut et al., 1964; Lee et al., 1978). In this study, both serum (0.889±0.008 and 2.512±0.225 ppm in breeding season and non-breeding season, respective-

ly) and seminal plasma (3.100±0.120 and 6.463±0.329 ppm in breeding season and non-breeding season, respectively) B concentrations were lower ($p<0.001$) in breeding season compared to non-breeding season. The higher levels of diet B concentrations in non-breeding season (53,56 ppm/ram/day) compared to breeding season (40,89 ppm/ram/day) may explain this difference to a certain degree. The dramatically high concentrations of B in non-breeding season may contribute to the lower semen quality. Like Cr, the exact mechanism of absorbtion from intestine, distribution and transfer from the circulating blood into the seminal plasma of B is unclear. The understanding of the role of these elements in non-breeding season in ram needs further investigations.

CONCLUSION

The determination and comparison of macro and trace elements in blood serum and seminal plasma in breeding and non-breeding seasons in ram, in this study, can bring a new perspective, which the breeding season of ram may reveal different levels of elements in semen for spermatogenesis. In this study, the higher levels of Cr and B in non-breeding season compared to breeding season both in serum and seminal plasma, regardless of diet intake suggest that these elements may play a crutial role on male fertility in Merino Ram.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

The healing effect of bone marrow-derived stem cells and aquatic activity in Achilles tendon injury

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ABSTRACT. The common treatment recommended for Achilles tendon rupture as the most common tendon injury during exercise is surgical intervention, while it eventually causes various clinical problems. This study assessed the healing effect of bone marrow-derived stem cells (BMSCs) and aquatic activities in Achilles tendon injury. Forty rats were randomly divided into 5 equal groups. Group 1 underwent aquatic activity, 72 h after a crush lesion formed on Achilles tendon, group 2 received 1×106 intra-articular BMSCs post-tendon injury, group 3 had aquatic activity together with BMSCs transplantation after tendon damage, group 4 just experienced tendon injury without any treatment intervention and group 5 was considered as the control group while did not undergo any tendon injury and did not receive any treatment measure. After 8 weeks, the animals were sacrificed and the tendons were transferred in 10% formalin for histological evaluation. There was a significant increase in fibroblast number in group 3 in comparison to other groups. However, there was a significant increase in collagen deposition in groups 2, 3 and 5 in comparison to group 1 and 4. A significant decrease was noted for cellularity in group 2 when compared to groups 1 and 4. Regarding tendon diameter in group 3; a significant healing was observed when compared to groups 2, 4 and 5. It was shown that aquatic activity together with cell transplantation was an effective therapeutic measure enhancing the healing in tendon injuries. These findings can open a window in sport medicine in treatment of tendon injuries.

Keywords: Aquatic activity, Bone marrow, Mesenchymal stem cells, Healing, Tendon.

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INTRODUCTION

The common treatment recommended for Achilles tendon rupture as the most common tendon injury during exercise is surgical intervention, while it eventually causes various clinical problems (Rodeo et al., 2010). During the recent decade, it was shown that biological factors may accelerate tendon healing in damaged tendons (Rajabi et al., 2015). Although many studies have indicated that exercising and physical activity is among effective factors in increasing tendon strength, the question whether physical activity after the surgery could accelerate the recovery of tendons biomechanical properties and lead to fundamental changes in healing process have still remained controversial (Rajabi et al., 2015).

Several researchers have mentioned the significant role of mesenchymal stem cells (MSCs) in tendon healing (McGoldrick et al., 2017; Romero et al., 2017). MSCs have been isolated from different tissues including bone marrow (BM) (Aliborzi et al., 2015), adipose tissue (Mehrabani et al., 2015a), menstrual blood (Faramarzi et al., 2016), dental pulp (Mehrabani et al., 2017), and endometrium (Shamosi et al., 2017). They have osteogenic (Shaterzadeh Yazdi et al., 2015), adipogenic (Mehrabani et al., 2015b) and neurogenic (Razeghian Jahromi et al., 2015) differentiation properties with similar features such as favorable proliferative capability, self-renewal, and differentiation potential (Mehrabani et al., 2013). Bone marrow-derived stem cells BMSCs) have opened a new window in regenerative medicine and cell transplantation purposes getting help from tissue engineering too (Hosseinkhani et al., 2014).

The beneficial effects of MSCs in healing of Achilles tendon injuries were previously reported demonstrating that use of MSCs could improve the biological and mechanical parameters of the tissue (Chong et al., 2007). Therefore, this study was conducted to determine the healing effect of a combination of BMSCs and aquatic activities in Achilles tendon injuries in experimental rat model.

MATERIAL AND METHODS

Forty male Sprague-Dawley rats weighing 200±20 g and aging 3 months were provided from Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran and housed in groups of 4 in transparent polycarbonate cages with dimensions of 54×18×18 cm at an environment of 21±2°C with the lighting of 12:12 light-to-dark ratio; light at 7:30 AM in 50±5 percent humidity. All procedures were done based on laws of Animal Care by Iran Veterinary Organization. The rats were randomly divided into 5 equal groups.

Group 1 underwent aquatic activity, 72 h after a crush lesion formed on Achilles tendon using a mosquito hemostat (Akinbo et al., 2008), group 2 received 1×106 intra-articular BMSCs post-tendon injury, group 3 had aquatic activity together with BMSCs transplantation after tendon damage, group 4 just experienced tendon injury without any treatment intervention and group 5 was considered as the control group while did not undergo any tendon injury and did not receive any treatment measure.

In order to prepare BMSCs, 5 rats were euthanized and under sterile conditions, femoral and tibial bones were removed while, the muscular and connective tissues were later detached. Under sterile conditions, both ends of the bones were cut and the BM was flushed out using a 10 ml syringe filled with Dulbecco's Modified Eagle Medium (DMEM; Biovet, Bulgaria) and 1% penicillin streptomycin (Sigma, USA). BM was then transferred on ice to the stem cell laboratory, Shiraz University of Medical Sciences, Shiraz, Iran.

The BM was diluted in an equal volume of DMEM, and centrifugation was undertaken at 1200 rpm for 7 minutes. After removal of the supernatant, the precipitate was transferred in 25 cm2 flasks containing DMEM supplemented with 10% fetal bovine serum (FBS; Biovet, Bulgaria), 1% L-glutamine (Sigma, USA) and 1% penicillin and streptomycin and the culture flasks were placed in CO2 incubator at 37°C with 5% CO2 and saturated humidity while the medium was replaced every 3 days. The adherent cells were subcultured at 80% confluency, by washing twice with PBS (Gibco, USA) and adding 0.25% trypsin (Gibco, USA) for 3 minutes. To inactivate the trypsin activity, equal volume of DMEM was added. Cell passage was continued until passage 5 and at each passage, the cells were counted too. Cell morphology was assessed by inverted microscope (Olympus, USA) as described by Gashmardi et al. (2017).

To assess the osteogenic differentiation property, BMSCs from passage 5 were seeded into 6 well plates and at 80% confluency, the cells were cultured for 21 days with low glucose DMEM containing 100 nM dexamethasone (Sigma, USA), 0.05l M ascorbate-2-phosphate (Wako Chemicals, USA), 10 mM b-glycerophosphate (Sigma, USA), 1% penicillin/streptomycin and 10% FBS. The medium was changed every 3 days. After 3 weeks, osteogenic differentiation was evaluated with Alizarin Red (Sigma, USA) staining (Gashmardi et al., 2017).

RT-PCR was done to determine the expression of MSC markers. So after extraction of the total RNA using the column RNA isolation kit (Denazist-Asia, Iran) based on manufacturer's instructions, it was evaluated by spectrophotometry. The complementary DNA (cDNA) was provided by AccuPower Cycle Script (RT PreMix Kit Bioneer, Korea) upon manufacturer's instruction. For each reaction; 15 µL of total RNA was used to reach a volume of 20 µL with the DEPC water. Twelve thermal cycles were conducted including 30 s at 20˚C for primer annealing, 4 min at 42˚C for cDNA synthesis, and 30 s at 55˚C for melting secondary structure and cDNA synthesis and 5 min at 95˚C for inactivation (Rahmanifar et al., 2016).

Then, 1 µL of template cDNA and PCR buffer, H2O, dNTPs, MgCl2, Taq DNA polymerase, and forward and reverse primers were mixed. The microtubules containing 20 µL of the mentioned mixture were transferred to a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) and 30 amplification cycles were performed (30 s denaturation at 95˚C, 30 s annealing at 64°C, 62°C, and 61°C and 30 s extension at 72˚C with the 5 min at 95˚C for primary denaturation and 5 min at 72˚C for final extension). PCR products were assessed for defined bands by gel electrophoresis by DNA safe stain in 1.5% agarose gel medium. The bands were seen by UV radiation and a gel documentation system (UVtec, Cambridge, UK) and photography was done.

Aquatic activity was undertaken for 8 weeks (five sessions per week). For adaptation, rats were put into a glass aquarium with the dimensions of 50×40×50 cm filled with 34°C water for 5 minutes for 3 days. Then the aquatic activity was extended for 8 weeks 5 days per week (5-30 minutes each session, Table 1) as described by Hart et al. (2001) and McVeigh et al. (2010). After 8 weeks, the animals were euthanized and sacrificed and the Achilles tendon was removed and transferred into 10% formalin buffer for histological evaluation using hematoxylin and eosin (H&E) staining. The studied factors in histological evaluation were the number of fibroblasts, collagen deposition, cellularity and tendon diameter. For statistical analysis, SPSS software (version 16, Chicago, IL, USA) was used applying Kolmogorov Smirnov, one-way ANOVA and Tukey post hoc tests. The significance level was considered a p value less than 0.05.

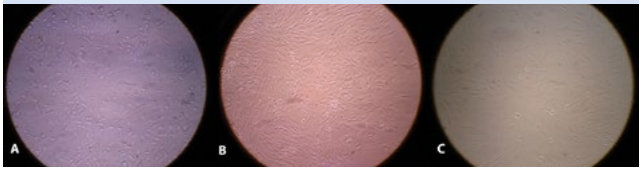
Table 1. Eight-week incremental aquatic activity program of studied rats.

Week	Stage
	Introduction stage: In this stage, to have optional activity, rats were put into a glass aquarium
1	With the dimensions of 50×40×50 cm filled
2	with 34°C water for 5 minutes for 3 days.
3	Aquatic activity: 5 min: Overload Stage:
4	days 1 to 5
5	days 1 to 5: Aquatic activity: 10 min
6	days 1 to 5: Aquatic activity: 15 min
7	days 1 to 5: Aquatic activity: 20 min
8	days 1 to 5: Aquatic activity: 25 min
	days 1 to 5: Aquatic activity: 25 min
	days 1 to 5: Aquatic activity: 30 min
	days 1 to 5: Aquatic activity: 30 min

RESULTS

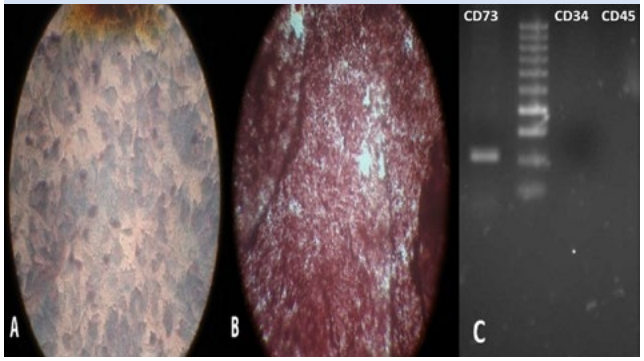
BMSCs were plastic adherent and fibroblast-like throughout all passages (Figure 1A-C). Culture of BMSCs in osteogenic media resulted into osteogenic differentiation of these cells due to presence

Figure 1. BMSCs were fibroblast-like throughout all passages. A: Passage 1, B: Passage 2 and C: Passage 3.



of calcium deposits after three weeks verified by Alizarin Red staining (Figure 2A, 2B). They showed

Figure 2. The osteogenic differentiation of BMSCs by Alizarin Red staining. A: Control, B: Osteogenic induction). C: The positive expression of CD73 marker confirming the mesenchymal stem cell property and negative expression of CD45 for markers of hematopoietic stem cells.



positive expression of CD73 marker confirming the mesenchymal stem cells and absence of CD34 and CD45 as markers for hematopoietic stem cells (Figure 2C). Table 2 shows the histological findings denoting to a significant increase in the number of fibroblast in group 3 in comparison to other groups (Figure 3A). A significant increase was visible in collagen deposition in groups 2, 3 and 5 in comparison to group 1 (p=0.001) and 4 (p=0.001,

Figure 3. The mean and standard deviation of Fibroblast numbers (A), Collagen deposition (B), Cellularity (C) and Tendon diameter (D) were shown in different groups. *P<0.05.

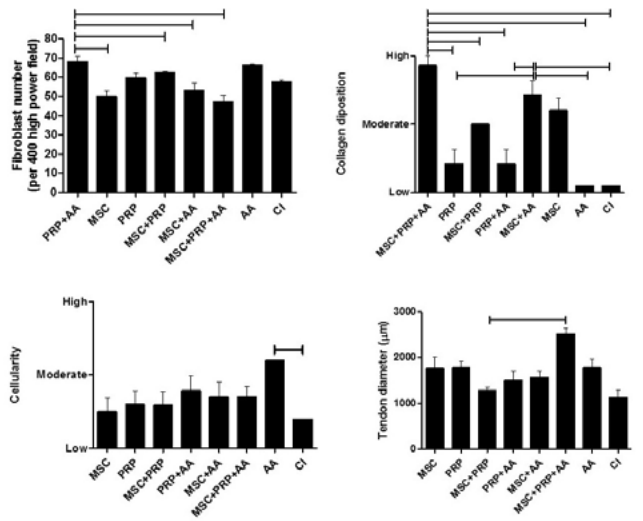
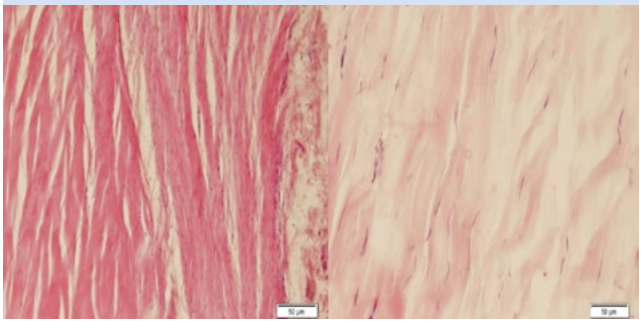


Figure 3B). There was a significant decrease in cellularity in group 2 compared to groups 1 (p=0.009) and 4 (p=0.04, Figure 3C).

Regarding tendon diameter revealing healing process, in the group receiving BMSCs and undergoing aquatic activity, an increase in tendon diameter was seen compared to the group just underwent cell transplantation (p=0.04) and the group with tendon injury and any treatment measure (p=0.01) and the control group with no tendon injury (p=0.04, Figure 3D). Figure 4 shows a moderate healing in Achilles tendon with collagen deposition in group 3.

Figure 4. Moderate healing in Achilles tendon with collagen deposition.



It shows that BMSCs together with aquatic activity could increase the collagen deposition compared to other groups while the difference was statistically significant too.

DISCUSSION

Our findings suggested that transplantation of BMSCs along with aquatic activities could be effective in repair of Achilles tendon injuries. The histological factors in assessment of healing of Achilles tendon injuries showed that the number of fibroblasts in transplantation of BMSCs along with aquatic activities had a statistically significant higher healing property in comparison to other groups. In response to tendon damage, fibroblasts migrated to the inflamed wound area together with type III collagen which later was substituted by type I collagen (Lisa and Zena, 2002). It was shown that the mechanical pressures due to the physical activities affected the collagens homeostasis that increased in the injured tissues, such as tendons and ligaments (Heinemeier, 2007). Injection of BMSCs along

Table 2. Mean and standard deviation of effective factors related to the healing of Achilles tendon in studied groups.

Variables Group	Fibroblasts	Collagen deposition	Cellularity	Tendon diameter	Total
1: Aquatic activity	51.25±10.8	1±0.0	2±0.0	1704.78±412.1	1759.03±408.6
2: BMSC transplant	48.75±7.9	2.5±0.75	1.2±0.46	1589.9±307.5	1642.49±308.5
3: BMSC transplant +Aquatic activity	65.25±2.9	2.5±0.53	1.6±0.51	2187.32±689.9	2256.7±690.39
4: Tendon injury without treatment	50.75±6.3	1±0.0	1.87±0.35	1485.97±307.08	1539.6±310.5
5: Control with no tendon injury	51.62±5.2	2.87±0.35	1.5±0.53	1582.33±128.37	1638.33±12.13

with aquatic activities or injection of BMSCs alone was demonstrated to increase collagens deposition significantly in comparison to injury group. Physical activity was shown to increase the number of fibroblasts and as a result, the collagen deposition.

Tendons are considered as facilitators of force transfer and the arrangement of tendon fibers is important in passive forces and pressures absorbing, as tendon strength is dependent on orientation and length of collagen fibers and consistency of transplanted tendons. The collagen and transplanted tissue metabolism was shown to be influenced by the amount of physical activity. Physical activity was demonstrated to increase the differentiation and change of the transplanted tissue in the tendons denoting to the physiological adaptations and repair of the damages on the extracellular matrix based on physical activities (Kjaer, 2004). In our study, injection of cells alone lead to an increase in collagen deposition, which substituted various cellular sources for tendon repair. Similarly, the role of MSCs in tendon repair was shown before, due to their high proliferation capacity and differentiation properties (Harris et al., 2004).

We reported a significant increase in cellularity in the group undergoing aquatic activity, compared to the control group that underwent just injury. Platelet rich plasma (PRP) was identically used to increase the performance and cellularity of the tendons and the amount of collagens and cellularity that were due to the increase in the metabolic activity and its positive impact on tissue repair (Lane et al., 2013). Our findings are also in accordance with the aforementioned study on the increase in cellularity.

We showed that the tendon diameter in the group undergoing aquatic activity together with cell transplantation revealed a significant increase. Similarly, it was also demonstrated that physical activity on treadmill for a week could increase the size of collagen fibers and tendon diameter in rats (Michna and Hartmann, 1989). In another study, an intense endurance exercise was found to increase in collagen deposition in Achilles tendon (Curwin et al., 1988). The physical exercise was noted to increase several growth factors in Achilles tendon which could stimulate the collagen synthesis and cell proliferation (Wang, 2006).

Rajabi et al. (2015) in their study on the healing effects of aquatic activities and allogenic injection of PRP on injuries of Achilles tendon based on

the number of fibroblast, cellular density, collagen deposition, and tendon diameter, showed that aquatic activity together with PRP injection was the therapeutic measure of choice enhances healing in tendon injuries. Godwin et al. in their study on implantation of BMSCs in horse with overstrain injury of the superficial digital flexor tendon showed that cell implantation was safe and appeared to reduce the re-injury rate (Godwin et al., 2012). Lacitignola and colleagues (2008) in cell therapy for tendinitis in horses by BMSCs together with exercise program stated that re-injury rate reduced and good to excellent outcome in term of athletic success were noted.

He et al. (2015) by applying BMSCs revealed attenuated adhesions in the early time point following flexor tendon repair and healing in rabbit flexor tendon. Human adipose stem cells were also shown as another cell source as a potential approach for tendon tissue repair (Vuornos et al., 2016). Transplantation of tendon-derived stem cells pre-treated with connective tissue growth factor and ascorbic acid as a different source of cells was found to promote better tendon repair in a patellar tendon window injury rat model (Lui et al., 2016). Umbilical cord blood-derived mesenchymal stem cells in a rabbit model with full-thickness rotator cuff tendon tear demonstrated the effectiveness of local injection of MSCs into the rotator cuff tendon to be a useful conservative treatment for full-thickness rotator cuff tendon tear repair (Park et al., 2015).

CONCLUSION

Identical to many studies that did not report pain for experimental animals, this limitation existed in our study too. We showed that injection of MSCs together with the aquatic activity could promote healing process that may be due to accumulation of monocytes, proliferation and differentiation of stem cells, migration of fibroblasts and angiogenesis. So based on our findings on the number of fibroblast, cellularity, collagen deposition, and tendon diameter, it was shown that aquatic activity together with MSC injection was a therapeutic of choice enhancing the healing of tendon injuries that can open a window in treatment of damages to tendons.

Disclosure of potential conflicts: All authors declare no financial or other conflicts of interest.

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CONFLICT OF INTEREST STATEMENT

No potential conflict of interest relevant to this article was reported.

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Research article
Ερευνητικό άρθρο

Factors affecting the prevalence of ticks in cattle and acaricidal activity of *Nicotiana tabacum* extracts

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ABSTRACT. The present study was designed to determine factors affecting the prevalence of ticks *(Hyalomma and Rhipicephalus)* in cattle in district Loralai of Balochistan and to evaluate the acaricidal activity of chloroform and methanol extracts of tobacco *(Nicotiana tabacum)*. A total of 670 cattle of different breeds, age and gender were examined for tick infestation with overall prevalence of 21.49% in Loralai. Friesian was more infected (26.15%) as compare to non-descriptive (22%) and Sahiwal (12.80%) breeds. Similarly, cattle less than one year old were most infected (27.90%) followed by those between 1-2 year (26.88%); the least prevalence was in cattle more than 2 years of age (19.34%). Higher prevalence was noticed in female cattle (21.98%) as compare to male cattle (16.92%). Three concentrations of *(Nicotiana tabacum)* (12.5mg/mL, 25mg/mL and 50mg/mL) were prepared in chloroform and methanol. The acaricidal activity of these extracts was determined by egg laying index and percentage inhibition of egg laying. The decline in egg laying index was significantly more by chloroform extract (10.048%, 17.378% and 25.143%) as compare to methanol extract (6.367%, 13.152% and 20.827%). Hatchability of eggs in chloroform extract was less than that in methanol extract (67.5%, 43.5% 17% and 77.5%, 47.5% and 23%) respectively. We concluded that the prevalence of ticks in cattle is affected by their age, breed and gender and that chloroform extract of *Nicotiana tabacum* is more acaricidal as compared to the methanol extract.

Keywords: Egg laying index, Hatchability, *Nicotiana tabacum* extracts, Ticks.

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INTRODUCTION

Ticks act as a vector for various diseases of livestock including the zoonotic diseases. In Pakistan, many species of ticks have been reported to affect livestock and decrease their production (Sajid et al., 2008). Keeping in mind the importance of different types of ticks present in different geographical distribution there are many reports about the types of ticks present in different areas of Pakistan. However, there is not a single study about the types of ticks present in Lorali District of Balochistan. The geographical location of Lorali in centre of Balochistan and its livestock population makes this area important for evaluation of ticks types and factors affecting the prevalence of ticks in this area. This laid the basis for the present study.

Another objective of present study to evaluate acaricidal activity of *Nicotiana tabacum* plant extracts for the control of ticks. As the control of ticks primarily depends upon synthetic acaricides (Adenubi et al., 2018) but it has been complicated due to drug resistance (Banumathi et al., 2017). The development of resistance and presence of drug residues in milk, meat, and the environment has created the need for search of less toxic substances for tick control. Keeping in view the above-mentioned facts there is a dire need to investigate alternative acaricides and new approaches for tick control.

The use of herbal plants against ticks is getting popular in developing countries (Wu et al., 2017). A number of easily available herbal plants have been evaluated for their acaricidal effect in Pakistan (Tabassum et al., 2008). Zaman et al. (2012) explained the synergistic effect of *Nicotiana tabacum* leave extract in combination with three other plants against ticks. In India Chaudhary et al. (2004) studied the in vitro effect of *Nicotiana tabacum* aqueous extract on Rhipicephalus haemaphysaloides ticks. As there have been no studies to evaluate the chloroform and methanol extract of *Nicotiana tabacum* against ticks therefore we evaluated the acaricidal effect of methanol and chloroform extract of *Nicotiana tabacum* plant. This research was particularly designed to study the effect of *Nicotiana tabacum* against most commonly occurring ticks district Loralai, Balochistan. We also hypothesize that breed, age and gender could be the most important determinants to infestation of ticks and evaluated the link of these parameters on tick prevalence.

MATERIALS AND METHODS

The prevalence of tick infestation and associated risk factors were also determined in 670 cattle in three tehsils of district Loralai including Bori, Dukki, Makhtar and their villages. The breed, age and sex of cattle were studied as associated risk factors for tick prevalence.

The *Nicotiana tabacum* plant was collected from the area of district Loralai and dried for 8 to 10 days. The leaves were grinded mechanically into powder form in Soxhlet apparatus (Iqbal et al., 2005) and stored in refrigerator at 4°C until used. The powder extracts were used to make three different concentrations of 12.5, 25 and 50mg/mL for both extracts (chloroform and methanol). The results from these concentrations were compared to positive and negative control groups. The buffer PBS used as negative control.

Adult immersion test (AIT)

The female engorged ticks collected from study area were washed with phostphate buffer saline (PBS) and dried by using paper towel. After that the ticks were weighed and then immersed into the formulated solution for five minutes. After immersion, ticks were incubated at 30ºC with relative humidity 80-90%. After that the ticks were placed in incubator for oviposition for (16-18 days). The reproductive index (RI) and % age Inhibition of oviposition (IO%) was calculated on the basis of following parameters (Sabatini et al., 2001).

- Mortality was recorded up to 14 days post treatment (dpt) when normal ticks complete egg laying.
- The egg masses laid by the live ticks were recorded.
- Reproductive index (RI) = egg weight (EW) engorged female weight (IFW).
- Percent Inhibition of oviposition (IO%) =RI control−RI treated RI control×100

Egg hatchability test

Approximately 10mg (200 embryonated eggs) were treated in each concentration for five minutes. After that ticks were incubated at 30ºC with relative humidity 80-90% until the eggs hatched (Ribeiro et al., 2008). Hatchability percentage was calculated as the number of hatched larvae divided by the total number of incubated eggs.

Egg laying index (IE) = mean weight of eggs laid (g)/ weight of females (g)

% inhibition of egg laying = IE control group - IE treated group/ IE control group x 100

Statistical analysis was made by SPSS version 22.0 using mean ± S.D. Qualitative variables were presented with help of frequency tables, pie charts and bar charts. Comparative analysis was done using one-way ANOVA and P value <0.05 was considered significant.

RESULTS AND DISCUSSION

The results of present study revealed that tick infestation was common in cattle of district Loralai, Baluchistan with 21.49% prevalence. Out of total 670 cattle 526 were positive for ticks. We found increased incidence of Hyloma ticks (380) as compare to Rhipicephalus (146). The geographical importance of district Loralai, Balochistan cannot be neglected due to its central position in Balochistan province. To the best of our knowledge this is the first study about the tick prevalence in Loralai, Balochistan. This prevalence is much higher than the tick prevalence in Quetta city of same province reported as low as 10% by Kakar et al. (2008).

Our results are different from another study conducted in Peshawar district (KPK) where Manan et al. (2007) found four types of ticks including Boophilus (46%), Hyalomma (31%), Rhipicephalus (18%) and Amblyomma (5%) in cattle with overall prevalence of 20%. However, our results are in agreement with the studies of Sajid et al. (2008) who found highest prevalence of Hyalomma followed by Rhipicephalus in cattle of district Layyah and Muzaffargarh.

The cross tabulation test showed significant difference (P<0.05) in prevalence of tick infestation in different cattle. Friesian was significantly more infected (P<0.05) (26.15%) as compare to non-descriptive (22%) and Sahiwal (12.80%) breeds. The more tick resistance of Sahiwal cattle (P<0.05) may be due to their thick leathery and naturally shivering skin. However, this is not in agreement with previous studies conducted in Lahore, Pakistan (Sadaqat et al., 2016) that reported highest tick infestation in Sahiwal (23.8%) followed by Friesian (16.3%) and non-descript cattle (12.8%) respectively. However, these differential results in both studies could be due to the difference in geographical location, sample size and different seasons.

Similarly, the cattle less than one year were most infected (27.90%) followed by cattle between 1-2 years (26.88%) of age. The least prevalence was in cattle more than 2 years (19.34%). This may be linked with development of immunity with increasing age. This is in agreement with the studies of Rehman et al. (2017) who described increased tick prevalence in older animals as compare to younger.

Higher prevalence was noticed in female cattle (21.98%) as compare to male cattle (16.92%). The production and reproduction of female may be important risk factors involved in high incidence of disease in female. This is in contrast with the studies of Rehman et al. (2017) that indicated almost three times more tick prevalence in male cattle (60.77%) as compare to female cattle (20%).

Acaricidal effects of chloroform extracts compared with control group

The ticks in group A1, A2 and A3 were treated in vitro with chloroform extract at the concentration of 12.5mg/mL, 25mg/mL and 50mg/mL respectively. The egg laying index ± SD calculated was 0.4782800±0.02789077, 0.4388300±0.05119868 and 0.3963600±0.03380405. In Post Hoc LSD multiple comparison tests the results of group A were significantly different (P<0.05) from the ticks in group C (untreated control group) with egg laying index± SD 0.5331200±0.02757486 (Table 1). The percentage inhibition of egg laying was group A1, A2 and A3 10.048, 17.378 and 25.143 in respectively.

Acaricidal effects of methanol extracts compared with control group

The ticks in group B1, B2 and B3 were treated in vitro with methanol extract at the concentration of 12.5mg/mL, 25mg/mL and 50mg/mL respectively. The egg laying index± SD calculated was 0.4991200±0.00948646, 0.4614300±0.03917896 and 0.4205800±0.04183098. In Post Hoc LSD multiple comparison tests the results of group B were significantly different (P<0.05) from the ticks in group C (control) with egg laying index± SD

Table 1: Least significant difference and multiple comparison test values of group A compared to other treated and untreated groups.

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Index of egg laying 12.5mg/mL of chloroform extract	Index of egg laying 12.5mg/mL of methanol extract	-.02084000	.01574539	.190	-.0523046	.0106246
	Index of egg laying 25mg/mL of methanol extract	.01685000	.01574539	.289	-.0146146	.0483146
	Index of egg laying 50mg/mL of methanol extract	.05770000*	.01574539	.001	.0262354	.0891646
	Index of egg laying 25mg/mL of chloroform extract	.03945000*	.01574539	.015	.0079854	.0709146
	Index of egg laying 50mg/mL of chloroform extract	.08192000*	.01574539	.000	.0504554	.1133846
	Control untreated	-.05484000*	.01574539	.001	-.0863046	-.0233754

0.5331200±0.02757486 (Table 2 & 3). The percentage inhibition of egg laying in group B1, B2 and B3 was 6.367, 13.152 and 20.827% respectively.

The previous studies have already explained the better anthelminthic effect of alcoholic extract of Nicotiana tαbacum as compare to aqueous extract. (Nouri et al., 2016). But the present study for the first time compared the effect of alcoholic extracts (methanol and chloroform) against ticks.

Bioactive compounds from *Nicotiana tαbacum* most-

Table 2: Least significant difference and multiple comparison test values of group B compared to other treated and untreated groups

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Index of egg laying 12.5mg/mL of methanol extract	Index of egg laying 25mg/mL of methanol extract	.03769000*	.01574539	.020	.0062254	.0691546
	Index of egg laying 50mg/mL of methanol extract	.07854000*	.01574539	.000	.0470754	.1100046
	Index of egg laying 12.5mg/mL of chloroform extract	.02084000	.01574539	.190	-.0106246	.0523046
	Index of egg laying 25mg/mL of chloroform extract	.06029000*	.01574539	.000	.0288254	.0917546
	Index of egg laying 50mg/mL of chloroform extract	.10276000*	.01574539	.000	.0712954	.1342246
	Control untreated	-.03400000*	.01574539	.035	-.0654646	-.0025354

Table 3: Least significant difference and multiple comparison test values of group C compared to other treated groups

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control untreated	Index of egg laying 12.5mg/mL of methanol extract	.03400000*	.01574539	.035	.0025354	.0654646
	Index of egg laying 25mg/mL of methanol extract	.07169000*	.01574539	.000	.0402254	.1031546
	Index of egg laying 50mg/mL of methanol extract	.11254000*	.01574539	.000	.0810754	.1440046
	Index of egg laying 12.5mg/mL of chloroform extract	.05484000*	.01574539	.001	.0233754	.0863046
	Index of egg laying 25mg/mL of chloroform extract	.09429000*	.01574539	.000	.0628254	.1257546
	Index of egg laying 50mg/mL of chloroform extract	.13676000*	.01574539	.000	.1052954	.1682246

ly include alkaloids. In present study it was noted that chloroform extract showed better acaricidal activities against *Rhiphicephalus* and *Hyloma* ticks as compare to methanol extract. Although methanol is more commonly used for good extraction various bioactive compounds due to its amphiphilic properties but it might be possible that alkaloids react better with chloroform as compare to methanol.

Hatchability of each treated group was calculated by dividing the number of eggs hatched with number of egg laid. Hatchability of group A1, A2 and A3 was 67.5%, 43.5% and 17% respectively. While for group B1, B2 and B3 it was 77.5%, 47.5% and 23% respectively. Hatchability of the ticks in group C (control group) not treated with any chemical only dipped in distilled water for five minutes was100%.

The egg laying index of ticks treated with lowest concentrations of both chloroform and methanol extracts of *Nicotiana tαbacum* was significantly less ($P<0.05$) than the control group. This could be due to active ingredients of *Nicotiana tαbacum*, which are helpful in controlling egg production in ticks. Chloroform extract with its highest concentration (50mg/mL) of the plant suppressed the hatchability of the egg to maximum extent but this difference was not significantly different ($P>0.05$) from the ticks treated with extracts of methanol.

CONCLUSIONS

The present study concluded that the use of methanolic and chloroform extract of *Nicotiana tabacum* plant is effective against ticks and egg laying characteristic of the tick is significantly inhibited by the extracts particularly chloroform extract. We also concluded that the tick prevalence is affected by various factors including age, breed and gender of infected cattle.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
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Toxoplasma gondii in sheep and goat livers: Risks for human consumption

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ABSTRACT. Toxoplasmosis is one of the most important zoonotic diseases worldwide and is caused by the protozoan *Toxoplasma gondii*. Besides vertical transmission during pregnancy, humans can become infected post-natally either by oral uptake of sporulated *Toxoplasma* oocysts or through ingestion of tissue cysts upon consumption of raw or undercooked meat. The aim of this study was to approximate the risk of human infection via liver consumption by estimating the seroprevalence and molecular prevalence of *T. gondii* in slaughtered sheep and goats in Iran. In the present study, livers from 150 sheep and 150 goats were collected at slaughter. In- house enzyme-linked immunosorbent assay was performed in *T. gondii* liver juice. Parasite-specific polymerase chain reaction was carried out on all samples obtained from liver tissues. Antibodies against *T. gondii* were detected by in-house ELISA in 32.6% sheep and 48% goat livers and 8% and 11.3% of sheep and goat livers were positive for the presence of *T. gondii* DNA, respectively. The results of this study provide baseline information on the presence of *T. gondii* in sheep and goats livers and imply an important human health and hygienic risk associated with the consumption of raw or undercooked liver from these animal species.

Keywords: *Toxoplasma gondii*, Liver, In house ELISA, PCR, Sheep, Goat

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INTRODUCTION

The apicomplexan parasite *Toxoplasma gondii* is a prevalent pathogen in wild and domestic animals worldwide, and is transmitted through the food chain by carnivorous feeding and scavenging (Dubey & Jones, 2008). It can be transmitted not only between intermediate and definitive hosts (sexual cycle) but also between intermediate hosts via carnivores (asexual cycle) or even between definitive hosts (Barros et al., 2018). Up to one- third of the human population in the world is chronically infected and toxoplasmosis has been considered one among the five parasitic diseases of priority for public health action (Montoya & Liesenfeld, 2004). Humans can become infected through three routes: (i) uptake of sporulated oocysts from the environment, (ii) consumption of raw or undercooked meat containing tissue cysts and (iii) pre-natal infection (Dubey, 2010). Infections in humans are primarily asymptomatic, but lymphadenopathy or ocular toxoplasmosis may occur in some patients. *T. gondii* infection in pregnant women may lead to abortion, stillbirth, or other serious consequences in newborns (Weiss & Dubey, 2009). In immunocompromised patients, toxoplasmosis can be fatal if left untreated, and the reactivation of a latent infection can cause life- threatening encephalitis (Machala et al., 2015). Among food animals, sheep and goats are well-known sources of human infection. The European Food Safety Authority (EFSA) has recognized toxoplasmosis as the parasitic zoonoses with the highest human incidence and has recently published a scientific opinion that clearly states the need of representative data on toxoplasmosis in Europe (EFSA, 2007). Furthermore, different options of obtaining *Toxoplasma*-free meat are being discussed since 2008 (Kijlstra & Jongert, 2008). *Toxoplasma* infection in sheep and goats at slaughter cannot be diagnosed because infected animals are asymptomatic and the cysts are too small to be visually detectable during meat inspection. Other rapid, reliable and cost-effective control methods for large-scale monitoring and surveys are therefore required. Serological testing of toxoplasmosis has been shown to be the most practical method for monitoring the exposure status of farms and the efficacy of the implemented control measures. Various serological methods, such as the modified agglutination test (MAT), immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), have been used to detect *Toxoplasma* IgG antibodies in blood and tissue samples (Basso et al., 2013). ELISA tests, which can be semi-automated, are cost-effective and convenient diagnostic tools for large-scale screening. Serological studies confirmed that meat juice serology has proven to be an excellent method of detection for *T. gondii* infection at slaughter in different species, including sheep, pigs, poultry, and has been proved to correlate well with serum serology (Basso et al., 2013; Meemken et al., 2014; Bacci et al., 2015). Several molecular tests to detect parasites have been developed in the last decade. Their specificity and sensitivity have gradually increased, and parasitic diseases that were previously difficult to diagnose using conventional techniques have begun to be identified by molecular techniques (Bastein, 2002). In some countries, people based on ancient beliefs consume liver for iron supplementation and elimination of anemia. Accordingly, pregnant women in Iran consume liver (especially undercooked) during their pregnancy. Due to the risk of *Toxoplasma* infection from undercooked liver consumption, the aim of the present study was therefore to evaluate Toxoplasma infection of sheep and goats liver using juice serological and molecular methods in livers, which can be conveniently collected at slaughter.

MATERIALS AND METHODS

Collection of samples

During a six month period (December 2016-May 2017), a slaughterhouse in the South-western part of Iran (Ahvaz, Khuzestan province) was visited once a week and liver samples were collected randomly from a total of 150 sheep and 150 goats. Immediately after opening the carcass, liver samples were obtained with sterile single use surgical blades and transferred into sterile plastic bags. A two-gram piece of each liver sample was aseptically transferred to a sterile 50 ml falcon tube and gently crushed using a sterile glass rod. Then 18 ml of sterile physiological saline solution was added to the falcon and vortexed for 20 min. The homogenates were filtered through two layers of sterile gauze and centrifuged at 4000 rpm for 30 min. The

supernatants were discarded, and the resulting pellets were suspended in 1 ml of sterile physiological saline solution and divided into two microcentrifuge tubes for serological and molecular study.

Liver juice serology

The assay was optimized using formalin-fixed *T. gondii* tachyzoites. The optimum dilutions were determined by checkerboard titration of antigen, serum and conjugate. Microplates (Greiner, Germany) were coated with 50 µL of tachyzoites of *T. gondii* RH strain (approximately 105 tachyzoites), diluted 1:50 in carbonate buffer (pH = 9.6), and then incubated overnight at 4°C. The plates were washed three times with 300 µL PBS containing 0.1% Tween 20 and blocked with 5% nonfat dry milk for 60 min at 37°C. After washing, 100 µL of liver juice sample, diluted 1:10 in PBS, was added and the microplate was incubated for 120 min at 37°C. After rinsing as above, 50 µL of alkaline phosphatase-labeled anti-goat/sheep conjugate (Sigma-Aldrich), diluted 1:500 in PBS was added to the wells, and then the microplates were incubated at 37°C for 60 minutes. Following washing, 50 µL of substrate solution (10 mg/mL 4-nitrophenylphosphate in 10 ml diethanolamine buffer, pH = 9.6) was added and the microplate was left for 30 min at room temperature. The reaction was stopped with 50 µL of 20% hydrochloric acid, and the optical density at 450 nm was read in an ELISA reader (ELX800-Biotec). The cutoff value of optical densities (OD) was determined by the method of Hillyer et al. (1992): the mean OD of negative control sera plus two standard deviations.

MOLECULAR STUDY

For DNA extraction, an aliquot of the resuspended pellet (500 µl) was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 5 min. The pellet was suspended again in 200 µl lysis buffer, stored at room temperature for 30 min and then heated at 100 °C for 10 min. Subsequently DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitatation with ethanol and resuspension in sterile distilled water. The extracted DNA was frozen until PCR analysis. For detection of T. gondii, primers targeting B1 gene were selected from the literature (Jalal et al., 2014). Primers used in the reaction were the forward primer with the sequence 5'-GAGACCGCGGAGCCGAAGTGC-3' and the reverse primer with the sequence 5'-CCTCCTCCTCCCTTCGTCCAAG-3', yielding a 469 bp product. All PCR were performed in 25 µl reactions, containing 12.5 µl Taq DNA polymerase master mix Red (Amplicon, Denmark), 1 µM primers and 50 ng DNA templates. PCR cycling included an initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 45s, annealing at 52ºC for 45s, extension at 72ºC for 60s. This was followed by a final extension at 72ºC for 5 min. PCR reactions included a negative control, consisting of the reaction mix and 2 µl of DNase/RNase-free water instead of DNA and a positive control consisting of DNA sample from the *T. gondii* tachyzoites (RH strain). PCR products were electrophoresed in 1.5% agarose (SinaClon Bioscience, Iran) in Tris–acetate–EDTA (TAE) buffer, stained with Green Safe stain (SinaClon Bioscience, Iran) and visualized under ultraviolet light. Positive samples showed a band of approximately 469 bp.

RESULTS

Based on the results of liver juice serology 49 sheep (32.6%) and 72 goats (48%) were positive for *T. gondii,* respectively. In 12 (8%) samples of 150 examined sheep livers, the PCR was positive and a band of approximately 469 bp was observed on the agarose gel which was considered as infection with *T. gondii* (Fig 1). Furthermore, parasite DNA amplification was obtained in 17 out of 150 (11.3%) goat livers. Table 1 represents frequency of *T. gondii* in sheep and goats livers as found by liver juice serology and molecular method.

After comparing the results of sheep liver juice serology and PCR, it was found that ten livers were positive in both methods while two livers were diagnosed PCR positive and serologically negative. Also, 39 livers were serologically positive but their PCR results were negative.

Comparing the results of goats' liver juice serology and PCR, it was demonstrated that 13 livers were serologically and molecularly positive for *T. gondii.* While four livers were only PCR positive, 59 livers were found positive only by juice serology method.

Table 2 represents results of liver juice serology and PCR in sheep and goats.

DISCUSSION

Foodborne diseases are caused by a number of agents, varying in severity from weak to chronic or acute disturbances that can affect or compromise the life of the consumer, and the agents of biological origin (bacteria, viruses, parasites) are the major cause of these diseases. Parasites, including *T. gondii*, are reported less frequently in humans, and have caused fewer outbreaks than bacteria and viruses. However, in many instances, their impact (severe illness, disability, death, and costs related to diagnostic procedures, hospitalization and treatment) on vulnerable groups of the population, and often in immunocompetent people, has been considerable (EFSA, 2007). Small outbreaks of toxoplasmosis have been associated with the consumption of raw meat in Korea, USA, France, French Guiana and New Zealand (Kijlstra & Jongert, 2008). Most farm animals that are naturally infected with *T. gondii* have been shown to carry infectious parasites in their meat. There are several new ready-to eat smallgoods which are meat products that may represent a source of *T. gondii* infection (Mie et al., 2008). In Iran, consumption of undercooked liver is common in pregnant women and since congenital toxoplasmosis can cause serious health problems in the fetus, the aim of this study was therefore to evaluate *Toxoplasma* infection in sheep and goats liver. Bioassay and molecular biology tests used to search for cysts in meat are complex and time consuming, due to the uneven distribution of cysts in the carcass, which often results in false negative results (Lundén et al., 2002). Antibodies usually reflect the exposure of the hosts to the parasite and could also reflect the infective status of meat (Dubey et al., 2008). ELISA is a large scale, simple and sensitive serological assay method that is useful for the surveillance and control of toxoplasmosis (Ferguson et al., 1989). This diagnostic test has been conducted for sanitary control using serum samples taken from slaughtered animals, despite presenting a sampling associated problems. This preventive approach is safe and useful, but frequently the only available sample for testing is already meat, which contains exudates, formed after the retail processing, and consisting mainly of blood and interstitial fluid. As reported elsewhere, there is a good correlation between ELISA results obtained for anti-*T. gondii* antibodies detected in meat juices and in serum samples (Lundén et al., 2002; Wingstrand et al., 1997). In the present study, an ELISA test was designed for detection of anti-*T. gondii* specific IgG in sheep and goat livers, using liver juices in order to determine the presumptive risk for consumers, acknowledging at the same time that seropositivity does not directly imply infectivity. Based on the liver juice ELISA results, 49 sheep (32.6%) and 72 goats (48%) were positive for *T. gondii*, respectively. Liver juice is a matrix easily available from sheep and goats at slaughter, and can also be used for the detection of other public health hazards such as Salmonellae, pathogenic Yersiniae and etc. Since it seems that the levels of *T. gondii* specific IgG are lower in liver juice compared to serum and to compensate this, a lower dilution factor was used for the liver juice samples. In accordance with the approach described by Wingstrand et al. (1997), who found excellent correlations for *Toxoplasma* antibodies between meat juice and serum, liver juice samples were 10 times less diluted than serum samples. Briefly, liver juice samples were diluted 1: 10. The overall estimate of seroprevalence of *T. gondii* in sheep and goats was generally high. Nevertheless, this count does not necessarily represent a hazard for the population as a seropositive animal does not compulsively harbour active tissue cysts with infective parasites (de A Dos Santos et al., 2005; Halos et al., 2010). For the parasite detection we used primers targeting B1 gene (a marker of *T. gondii*) as they were described as the sensitive ones even in tissues (Martínez-Flores et al., 2017). PCR can be considered as a useful method to assess *T. gondii* prevalence in tissues. Based on PCR results, parasite DNA was detected in 8% and 11.3% of sheep and goat livers in the present study. The presence of DNA shows that the meat originates from a *Toxoplasma*-infected animal but this does not necessarily mean that the product contains infectious organisms. In China, liver, lung and lymph nodes from 403 Yunnan black goats were

collected randomly from different administrative regions in Yunnan province, and B1 gene was identified using PCR in 20 (5%) animals (Miao et al., 2015).

After comparing the results of sheep liver juice serology and PCR, it was found that ten livers were positive in both methods while two livers were diagnosed PCR positive and serologically negative. Furthermore, 39 livers were serologically positive but their PCR results were negative. Comparing the results of goats' liver juice serology and PCR demonstrated that 13 livers were serologically and molecularly positive for T. gondii. While four livers were only PCR positive, 59 livers were found positive only by juice serology method. Table 2 represents results of liver juice serology and PCR in sheep and goats. Recent infection with no pervious exposure may explain negative ELISA samples with positive PCR results.

The distribution of *T. gondii* parasites within the same tissue is random, and parasite density may be low. Therefore, a negative result has to be interpreted carefully due to the possibility that the parasite could be present in unexamined parts of the target tissue. The mentioned explanation can be considered for positive ELISA samples with negative PCR results.

Finally, since there is a globalization in the trade of animals and food worldwide, rules of trading meat and meat products ought to guarantee that all imports and exports fulfill high standards that ensure food safety. These rules should also be extended to animal health status and high standards of meat and meat products in order to avoid human toxoplasmosis. Besides measures focusing on pre-harvest food safety (e.g. surveillance and monitoring in animals), post-harvest strategies at slaughter and during food processing have become increasingly important in recent years. With regard to meat processing, demands of consumers for pathogen free meat products have focused the attention of meat industry on food safety and the necessity to produce meat that is wholesome, safe, and of high quality, using the appropriate technological treatments. Furthermore, liver should be considered as a potent source for *Toxoplasma* infection.

CONCLUSIONS

Overall, the results of our study show that Toxoplasma infections are prevalent in sheep and goats livers. Pregnant women who are found seronegative for *T. gondii* and other susceptible categories of people in Iran should therefore be very careful when preparing and consuming liver and they should make sure that they strictly apply to the recommendations for food hygiene and safety, such as washing hands after the preparation of liver and consuming only well-cooked liver.

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CONFLICT OF INTEREST

There is no conflict of interest.

Figure 1. Amplication of *T. gondii* DNA. Lane M is a 100-bp ladder. Lane P is a positive control DNA lane. Lane N is a negative control DNA lane. Lanes 1,3 represent negative samples. Lanes 2 represents a positive sample.

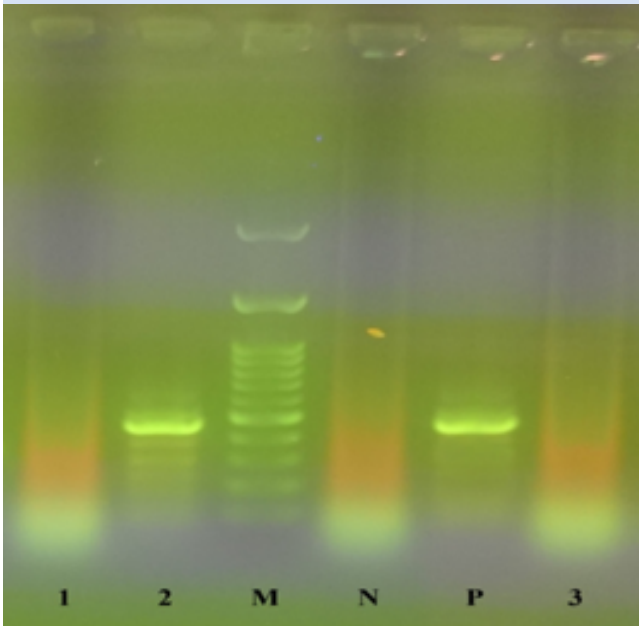

Table 1. Liver juice in- house ELISA and PCR results for *T. gondii* infection.

		Positive (%)	Negative (%)	No. examined
ELISA	Sheep	49 (32.6)	101 (67.4)	150
	Goat	72 (48)	78 (52)	150
PCR	Sheep	12 (8)	138 (92)	150
	Goat	17 (11.3)	133 (88.7)	150

Table 2. Comparison of results of liver juice serology and PCR in sheep and goats.

			Liver juice ELISA	
Test	Result	No/Total	+	-
Sheep- PCR	+	12/150	10	2
	-	138/150	39	99
Goat- PCR	+	17/150	13	4
	-	133/150	59	74

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Research article
Ερευνητικό άρθρο

Feeding a definite concentration of eicosapentaenoic and docosahexaenoic fatty acids to laying hens

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ABSTRACT. The experiment designed to study the influences of nutritional eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids (FA) on performance, egg yolk fat characteristics and FA profile in laying hens. From 30 to 36 weeks of age, 180 laying hens were allotted randomly to 2 dietary treatments, each of 6 replicates (15 birds for each replicate). The control diet was supplied with soy oil while the experimental diet was supplied with EPA and DHA to create 2 different ratio of n-6 to n-3 FA (18.8:1 and 5:1, respectively). The egg production % was recorded daily. The eggs were weighed to estimate egg mass. Also, feed consumption was recorded daily and the feed conversion ratio (FCR) was estimated. The FA profile of egg yolk was determined in the last week of the experiment. The dietary EPA and DHA resulted in significantly higher egg production (76.89 versus 67.23%), weightier egg mass (42.46 versus 37. 72 g) and lower FCR (2.49 versus 2.72) than the control. Also, supplying the dietary EPA and DHA was reflected in increasing of total polyunsaturated and n-3 FA in the eggs. Moreover, reducing the ratio of n-6 to n-3 FA to 5:1 decreased egg triglycerides, total cholesterol and cholesterol associated with low density lipoprotein and very low density lipoprotein. In conclusion, supplying EPA and DHA in the diet of laying hens positively influences performance, egg yolk FA profile and cholesterol. Practically, EPA and DHA could be used in laying hen diets to improve their performance and enhance public health of egg consumers.

Keywords: eicosapentaenoic, docosahexaenoic, laying hen, performance, egg yolk.

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INTRODUCTION

Hen eggs are an economic source of protein and fat for humans. But, there is a trouble owing to their cholesterol and fat content. Also, the high n-6 to n-3 FA ratio increases the problem of heart diseases (Simopoulos, 2006). The consumption of EPA and DHA FA is suboptimal, contributing to the problem of heart diseases in humans. The new qualified health claim for n-3 FA is to improve patient outcome measures (Food and Drug Administration, 2013). Animal nutrition has substantial ability to improve FA profile of animal products, reduce cholesterol content and decrease n-6 to n-3 ratio.

EPA and DHA have numerous health values. They are mainly related to cardiovascular diseases, central nervous system, mental health diseases and immune functions (Wassall and Stillwell, 2009; Riediger et al., 2009; Alexander et al., 2017). To our knowledge, the benefits of EPA and DHA for performance and health of laying hens are less investigated. This is the first paper used EPA and DHA with a definite concentration in laying hens diet and declare any deleterious effects of further substances associated with omega 3 FA sources.

Increasing the consumption of n-6 FA alters the FA profile of the phospholipids of cell membranes. Subsequently, increased linoleic and arachidonic acids which might alter gene expression and eicosanoid synthesis toward a pro-inflammatory state (Calder, 2012). Also, increasing n-3 FA consumption would increase these FA in the membrane phospholipids, which are expected to lessen inflammatory responses. So, it will be necessary to decrease the consumption of n-6 FA and increase the consumption of n-3 FA (Cottin et al., 2011).

The previous studies investigated the dietary supplementation with linseeds, linseed oil, algae bio mass, or fish oil. But in this experiment, a mixture of EPA and DHA was used with a definite dose to declare any deleterious substance which could affect hen performance. In this experiment, the design was towards fortification of hen eggs with EPA and DHA and studying its relation to egg yolk FA profile and cholesterol concentration. Therefore, the aim of this experiment was to study the influences of EPA and DHA supplementation on hen performance, lipid characteristics and FA profile in egg yolk.

MATERIALS AND METHODS

Laying hens management and diets

From 30 to 36 weeks of age, 180 Lohman brown laying hens were housed in the poultry experimental facility at the institute of Animal Health and Research, Tanta, Egypt, under standard conditions of lighting (16 h light), environmental temperature (25±2°C) and ventilation. The birds were randomly placed in 12 pens (15 hens per pen) containing deep litter of wood shavings equipped with round drinker, feeder and laying nest. The trial was designed with two dietary treatments and six replicates each (15 hens/replicate). Each diet consisted of constant basal components. In the experimental diet, the EPA and DHA supplement was added to obtain n-6: n-3 of 5:1. According to NRC (1994), the diets were formulated to be isonitrogenous and isocaloric (Table 1). During the experimental period, the feed was given as 110 g/hen/day, water was offered ad libitum.

Performance measurements

All birds were weighed individually at the start and the end of the experiment to determine the change in body weight. The eggs were recorded daily to calculate laying rate (%). The eggs weight and feed intake were determined to calculate feed conversion ratio and egg mass.

Chemical analyses of the eggs yolk

Eighteen eggs from each group were randomly collected. The egg yolk was separated from its albumen using a yolk separator, weighed and homogenized gently. The yolk samples were frozen at – 20 °C until used for analyses of triglycerides, cholesterol and FA profile. The total lipid of the egg yolk was extracted using the chloroform: methanol (2:1) method as explained by Folch et al., 1957. Yolk cholesterol concentration was determined according to Rotenberg and Christensen (1976) using cholesterol reagent and standard (Spinreact, Citra Coloma, Spain). FA profile of the egg yolk was analyzed according to Radwan (1978) using gas chromatograph (Hewlett Packard, 6890). The flame ionization detector and injector temperatures were set at 250 °C and 225 °C, respectively. The temperature was set from 130 °C to 225 °C at a rate 6 °C/min. Helium was used as a carrier

gas at a flow rate of 1 ml/min. A standard FA methyl ester mixture was used to identify all the FA peaks.

Table 1 Physical and chemical composition of the laying hen diets (%)

Ingredient %	Treatments*	
	Control	Omega 3
Corn grain, cracked	72.27	72.27
Soybean meal, without hulls	14.9	14.9
Corn gluten meal	3	3
Di calcium phosphate	0.76	0.76
Limestone	8	8
Salt	0.37	0.37
Methionine	0.03	0.03
Lysine	0.04	0.04
Premix†	0.3	0.3
Soya oil	0.33	-
EPA and DHA supplement‡	_	0.33
Chemical composition		
ME (Kcal/kg)	2910.9	2926
Crude protein %	15.34	15.34
Lysine %	0.69	0.69
Methionine %	0.32	0.32
Crude fat %	3.28	3.2
Linoleic acid %	1.82	1.66
EPA, DHA %	-	0.25
Calcium %	3.3	3.3
Available phosphorus %	0.26	0.26
Sodium %	0.15	0.15
Chloride %	0.27	0.27
Potassium %	0.53	0.53

*Treatments represent the control group which was maintained at n-6: n-3 ratio of 18.8:1 while in the omega 3 group, the ratio was 5:1.
†Provided per kilogram of diet: Retinol, 5500 IU; Cholecalciferol, 1,250 IU; Vitamin E (dl-alpha-tocopherylacetate), 12 IU; menadione, 2.5mg; riboflavin, 6 mg; calcium pantothenate, 8 mg; niacin,15 mg; pyridoxine 2 mg; folic acid, 1 mg; vitamin B12, 7μg; Mn, 50 mg; Zn, 55 mg; Fe 40 mg; Cu, 4 mg; I,2 mg; Co, 0.3 mg; ethoxiquin, 150 mg.
‡EPA and DHA supplement: (Berkley and Jensen) soft gel capsules composed of EPA and DHA 500 mg.

Statistical analysis

Variance of data was analyzed using the ANOVA procedure (Goodnight, 1979) for analysis of variance. Differences due to treatment were considered significant at $P < 0.05$.

RESULTS

Feed intake and laying performance

Feed intake did not significantly differ between the control and the omega 3 groups (Table 2). Subsequently, the intake of total FA was nearly similar (Table 2). But, intake of linoleic and total n-6 FA increased in the control group. While, the intake of EPA, DHA and total n-3 FA increased in the omega 3 group (Table 2). Body weight of hens in both groups remained nearly stable throughout the experiment (Table 2).

Table 2. Effect of dietary eicosapentaenoic and docosahexaenoic fatty acids on feed intake and laying hen performance.

Item	Treatment1		P-value
	Control	Omega 3	
Feed intake, g/d	102.4± 1.51	105.8±1.19	–
FA intake, g/d	3.02±0.04	3.06±0.04	–
Linoleic	1.86±0.12^{a}	1.75±0.07^{b}	*
EPA + DHA	0.0±0.0^{b}	0.27±0.01^{a}	**
Total n-6	1.63±0.1^{a}	1.51±0.06^{b}	*
Total n-3	0.08±0.01^{b}	0.33±0.01^{a}	**
Initial body weight (kg)	1.59±0.03	1.65±0.02	–
Final body weight (kg)	1.62±0.03	1.68±0.08	–
Body weight gain (kg)	0.03±0.04	0.03±0.08	–
Egg production, %	67.23±1.5^{b}	76.89±0.8^{a}	**
Egg weight, g	56.11±2.0	55.22±2.0	–
Egg mass, g	37.72±1.34^{b}	42.46±1.62^{a}	*
Feed conversion ratio	2.72±0.1^{a}	2.49±0.1^{b}	*

*Treatments represent the control group which was maintained at n-6: n-3 ratio of 18.8:1 while in the omega 3 group, the ratio was 5:1. Values±SE with different superscripts within a row represent differences among treatments; *P<0.05 or **P<0.01.

Egg production increased in the omega 3 group and this response was consistent throughout the study (Table 2; Figure 1). Also, FCR decreased in the omega 3 group (Table 2). Throughout the study, hens fed EPA and DHA FA had a consistently weightier egg mass output compared with those in the control group (Table 2).

Fatty acid profile of egg yolk

FA profile of egg yolk was significantly altered by dietary EPA and DHA (Tables 3). The saturated fatty acids (SFA) were decreased, while poly-unsaturated fatty acids (PUFA) were increased (P<0.05). Whereas no significant effect (P>0.05) in the contents of monounsaturated fatty acids (MUFA) (Table 3).

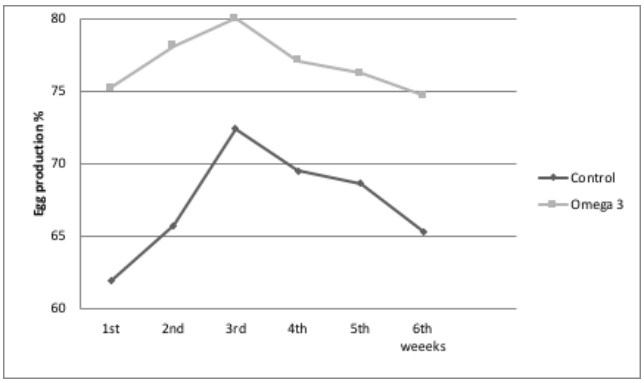
Table 3. Effect of dietary eicosapentaenoic and docosahexaenoic fatty acids on egg yolk fatty acids profile

Fatty acid, %	Treatment*		P-Value
	Control	Omega 3	
Myristic (C14:0)	0.54±0.03	0.51±0.02	–
Petadcaoic (C15:0)	0.38±0.02[a]	0.31±0.02[b]	**
Palmitic (C16:0)	24.38 ±0.49[a]	22.19 ±0.52[b]	**
Palmitoleic (C16:1)	3.57±0.16	4.08±0.1	–
Margaric (C17:0)	0.48±0.04[a]	0.29 ±0.04[b]	**
Stearic fatty acid (C18:0)	16.49±0.31[a]	12.33±0.37[b]	**
Oleic (C18:1)	39.88±0.54	40.22±0.55	–
Elaididic (C18:1 trans2)	0.75±0.04	0.67±0.03	–
Linoleic (C18:2n-6)	10.51±0.28	10.68±0.22	–
Linolenic (C18:3n-3)	0.72 ±0.04[b]	1.93 ±0.13[a]	**
Arachidonic (C20:4n-6)	0.92±0.02[a]	0.85±0.03[b]	*
EPA (C20:5n-3)	0.23±0.03[b]	1.52±0.06[a]	**
DPA (C22:5n-3)	0.46±0.02[b]	1.45±0.07[a]	**
DHA (C22:6n-3)	0.69±0.03[b]	2.97±0.13[a]	**
SFA	42.27±0.62[a]	35.63±0.67[b]	**
MUFA	44.20±0.65	44.97±0.56	–
PUFA	13.53±0.26[b]	19.40±0.28[a]	**
n-6 FA	11.43±0.28	11.53±0.21	–
n-3 FA	2.1±0.04[b]	7.87±0.16[a]	**

*Treatments represent the control group which was maintained at n-6: n-3 ratio of 18.8:1 while in the experimental group, the ratio was 5:1. Values±SE with different superscripts within a row represent differences among treatments; *P<0.05 or **P<0.01. EPA, eicosapentaenoic. DPA, Docosapentaenoic ;DHA, Docosahexaenoic; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

As it was hypothesized that increasing dietary EPA and DHA would increase them in the eggs of hens (Table 3) when compared with the control one. Subsequently, the ratio of n-6 to n-3 FA was decreased (P < 0.05) in egg yolk of the treated group. Although there was no significant effect on egg yolk linoleic acid concentration in both groups. Arachidonic acid was decreased (P<0.05) in omega 3 treated group.

Figure 1. Effect of dietary eicosapentaenic and docosahexaenoic fatty acids on egg production of laying hen.



Egg yolk lipid characteristics

Egg yolk weights of both groups remained unchanged during the experiment (Table 4). There were a significant decrease (P< 0.05) in the levels of egg yolk triglycerides, total cholesterol, VLDL cholesterol and LDL cholesterol in the n-3 FA supplemented group. While HDL cholesterol persisted unchanged in the two groups (Table 4).

DISCUSSION

Feed intake and change in body weight were not significantly different among diets. Similar results were published by Charlotte et al., (2013) who fed laying hens 5% and 10% microalgae supplement contained 1.5% EPA. In other study, feed intake and body weight were not changed in hens fed 2% deodorized menhaden oil, but feed intake only was decreased at a level of 4% (Gonzalez-Esquerra and Leeson, 2000). That was explained by the high metabolizable energy of menhaden oil than vegetable oil. In our study, the diets contained similar metabolizable energy (Table 1). In the current study, the egg production significantly increased (P<0.05) in hens fed diet contained EPA and DHA. Numerical increase (P>0.05 but <0.1) in egg production was

Table 4. Effect of dietary eicosapentaenoic and docosahexaenoic fatty acids on egg yolk lipids characteristics.

Item	Treatment*		P-Value
	Control	Omega 3	
Egg yolk weight (gm)	14.37±0.54	14.04±0.51	–
Triglycerides (mg/egg yolk)	157.6[a]±8.3	130.1[b]±6.3	*
Total cholesterol (mg/egg yolk)	166.1[a]±6.4	146.5[b]±5.6	*
Total cholesterol (mg/g egg yolk)	11.66±0.59	10.5±0.44	–
LDL mg/egg yolk	93.2 [a] ±5.9	78.4 [b]±6.2	–
HDL mg/egg yolk	41.32±1.8	42.02±0.94	–
LDL/HDL ratio	2.33±0.25	1.88±0.17	–
VLDL mg/egg yolk	31.51 [a] ±1.7	26.03 [b]±1.3	*

*Treatments represent the control group which was maintained at n-6: n-3 ratio of 18.8:1 while in the experimental group, the ratio was 5:1. Values±SE with different superscripts within a row represent differences among treatments; *P<0.05 or **P<0.01. LDL: low density lipoprotein. HDL: high density lipoprotein. VLDL: very low density lipoprotein.

reported by Lawlor et al., (2010) for hens fed 4% and 6% fish oil. Other trials in which flaxseeds were fed showed egg production remain unchanged (Bean and Leeson, 2003) or decreased (Aymond and Van Elswyk, 1995). Comparisons the current and the previous results in the literature, our results showed a positive effect of EPA and DHA than the other n-3 FA sources (fish and linseed oils) with respect to feed intake, palatability and egg production as mentioned previously. These differences might be due to the anti-nutritional factors in flaxseed which impair utilization of energy yielding nutrients (Bean and Leeson, 2003). Other factors influence egg production such as hen's strain and age (Scheideler et al., 1998), changes in diet composition (Gonzalez-Esquerra and Leeson, 2001) and feed formulation based on calculated energy value (Ilse et al., 2012).

Laying hens fed EPA and DHA had a weightier egg mass output compared with those fed the control diet. This indicated that supplementation of EPA and DHA increased feed efficiency in hens, probably as a consequence of altered nutrient partitioning by reducing inflammatory response (Komprda, 2012). This hypothesis will require further investigation of the anti-inflammatory effect of EPA and DHA in hens.

These results showed that the diet supplemented with EPA and DHA had no significant influence on egg weight, in agreement with other data (Caston and Leeson, 1990; Aymond and Van Elswyk, 1995; Ferrier et al., 1995; Bean and Leeson, 2003; Charlotte et al., 2013). Some researchers (Caston et al., 1994; Scheideler and Froning, 1996) observed a decrease in egg weight of hens fed flaxseed. Based on the literature, our results have shown the advantage of EPA and DHA over flaxseed as a supplier of n-3 fatty acid for laying hens. Caston et al. (1994) observed reduced metabolizable energy in hens fed diets augmented with flaxseed. Along with anti-nutritional factors in flaxseed which reduce absorption efficiency of energy yielding nutrients (Bean & Leeson, 2003).

Increasing PUFA/SFA ratio in eggs could reduce LDL-cholesterol level in serum of egg consumers (Grundy and Denke, 1990). Also, low SFA reduces development of heart disease and disorders related to blood vessels (Simopoulos, 1997). Concerning to the influences of EPA and DHA on egg yolk FA profile, our results revealed that there were a significant reduction in SFA (C18:0, C16:0, C17:0, C15:0), whereas the contents of MUFA were not altered. Egg yolk from hens fed EPA and DHA contained more UFA than those fed the control diet, indicating that saturation of FA was depressed by dietary EPA and DHA. The results of this experiment were nearly similar to the previous experiment of Cachaldora et al., (2008) who compared effects of various types of fatty acids in hen diets and concluded that fish oils increased EPA and DHA in egg yolk. While, soybean and linseed oil increased LA and ALA, respectively at the expense of monounsaturated fatty acids. As in previous studies (Aymond and Van Elswyk, 1995; Ferrier et al., 1995; Scheideler and Froning, 1996) who have been used flaxseed in hen's diet. They observed that yolk ALA increased proportionally to flaxseed amount. But, content of DHA increased to a smaller extent, not in lined response to flaxseed amount, and content of EPA hardly increased. The results confirms the connection between dietary lipids and egg lipid composition because

hens supplemented with EPA and DHA produced significantly eggs fortified with n-3 FA than those fed the control diet. Subsequently, the n-6/n-3 ratio has been decreased in that eggs. Similar findings have been reported by Yannakopoulos et al., (2005); Yalcyn et al., (2007). Ilse et al., (2012) has reviewed 26 publications concerned with n-3 FA in laying hens and observed that laying hens as well as human have limited ability to convert ALA into DHA or EPA. Previous and current data show the importance of feed supplementation with EPA and DHA to produce eggs fortified with n- 3 FA essential for human.

Hens Supplemented with EPA and DHA showed lower values of arachidonic acid ($P < 0.05$) than those fed the control diet which was consistent with the previous results of Cherian et al., 2007. Hens in the control group consumed more linoleic acid than those supplemented with EPA and DHA. Thus, our results indicated that, there was a positive relation between the consumption of linoleic acid and the denovo synthesis of arachidonic acid. Supplementation of EPA and DHA had a significant reduction of egg yolk triglycerides, total cholesterol, LDL-cholesterol and VLDL-cholesterol. This effect may be due to the influence of n-3 FA in increasing hepatic oxidation (Mashek et al., 2002). Also, it was reported that increasing intake of n-3 FA reduced fat deposition in spite of similar caloric intake which reinforces the influence of n-3 FA in nutrient partitioning (Buckley and Howe, 2010). Increasing consumption of n-3 FA increased concentration of these FA in liver cells, which increased hepatic expression of gluconeogenic enzymes (Bilby et al., 2006 and Do Amaral, 2008). Therefore, alteration of FA profile of hepatic tissue could influence metabolism and synthesis of other nutrients, consequently affect nutrient partitioning and favor egg production of hens. Moreover, Steinhilber, (2005), noticed that hens with high egg production rates have lower egg cholesterol level compared to those with low egg production rate. Ginzberg et al., (2000) found a reduction in blood cholesterol level but were incapable to decrease egg yolk cholesterol after feeding with red algal biomass for only 20 days. They hypothesized that longer feeding red algal biomass might decrease egg cholesterol level.

CONCLUSION

The ratio of n-6 to n-3 of 5 to 1 using EPA and DHA in the diet of laying hens improved egg production and efficiency of feed conversion into egg mass. These results were not on the expense of body weight which indicates that EPA and DHA favor nutrient partitioning for egg production.

The nutritional quality of eggs has been increased with respect to high PUFA/SFA ratio, low n-6/n-3 ratio, low triglycerides and low cholesterol content. Further research should be conducted to evaluate influences of EPA and DHA on hepatic metabolism and gene expression in hens.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

The investigation of xylazine, detomidine, isoflurane and sevoflurane anaesthetic combinations on clinical, laboratory and cardiovascular parameters and on intraocular pressure in horses

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ABSTRACT. The aim of this study was to investigate the effects of anaesthetic combinations of xylazine, detomidine, sevoflurane and isoflurane on clinical, laboratory, and cardiovascular parameters as well as their effects on intraocular pressure in horses. Twenty-four mixed-breed horses (twelve male and twelve female) were used for this study. The horses were allocated into four groups (six horses in each group): XS (xylazine-sevoflurane), XI (xylazine-isoflurane), DS (detomidine-sevoflurane) and DI (detomidine-isoflurane). Clinical evaluations, hematological, biochemical tests and measurement of intraocular pressure were done before (0th), during (5th, 15th and 30th min) and at the end of anaesthesia (60th min). The detected differences were statistically evaluated. In conclusion, this study shows that the anaesthetic combinations of sevoflurane and isoflurane with xylazine and detomidine provided safe and suitable anaesthesia in horses. Our study did not reveal any statistical differences in intraocular pressure measurements. However, it should be noted that intraocular pressures were measured with the animals lying down and our results do not rule out changes in intraocular pressures in a standing position. We concluded that these anaesthesia protocols are suitable for ophthalmic surgery.

Keywords: anaesthesia, equine, eye.

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INTRODUCTION

Alpha-2 (α-2) agonists are the greatest improvements in modern equine practice. For treatments and diagnostic procedures, α-2 agonists are widely used in equine medicine and anaesthesia (Mutoh et al., 1997; Guedes et al., 2017). Administration of α-2 agonists also reduces the amount of inhalational anaesthetic needed to maintain general anaesthesia in horses (Nyman et al., 2009; Rohrbach et al., 2009). α-2 Agonists such as detomidine, xylazine, medetomidine and romifidine are used for premedication in horses, and they have similar properties (Arıcan et al., 2015).

The inhalation anaesthetic agents such as isoflurane and sevoflurane are used for procedures requiring long-term anaesthesia in horses. Unlike injectable anesthetics, they enter the body through the lungs and move into systemic circulation from the respiratory tract (Hall et al., 2000; Del Barrio et al., 2017).

Isoflurane is an inhalant anesthetic agent with a low blood/gas solubility (1.4 in horses) coefficient. The lower blood/gas solubility causes rapid induction and short recovery time from general anaesthesia (Kalchofner et al., 2009; Ida et al., 2013). It leads to dose-dependent cardiac and respiratory depression. The α-adrenergic stimulation associated with isoflurane increases blood flow in skeletal muscles and reduces systemic vascular resistance and arterial blood pressure (Yamashita et al., 2006; Steffey, 2009). Sevoflurane is structurally similar to isoflurane but has lower blood/gas solubility (0.6 in horses). Its effects on the central nervous system (CNS) are fewer than those of isoflurane, but there is still a dose-dependent depression. CNS depression is greater at increasing doses when compared to the other inhalation agents. It also reduces cerebral vascular resistance and metabolism at high doses (Yamanaka et al., 2001; Kronen, 2003).

Intraocular pressure (IOP) is defined as the equilibrium between production and drainage of humor aqueous in the ciliary processes of the eye. The anaesthetic agents may cause changes in IOP (Komáromy et al., 2006; Monk et al., 2017). The aim of this study was to investigate the effects of xylazine, detomidine, isoflurane and sevoflurane anesthetic combinations on clinical, laboratory, and cardiovascular parameters as well as their effects on IOP in horses.

MATERIALS AND METHODS

The current study was performed in the Surgery Department of the Faculty of Veterinary Medicine, University of Selcuk, Turkey and was approved by the Ethics Committee of the Faculty of Veterinary Medicine. Twenty-four mixed-breed horses (twelve male and twelve female) weighing 450 ± 51 kg, 7.6 ± 5 years old were used for this study. The clinical examination and hematological tests were done before starting the study. The horses were allocated into four groups (six horses in each group): XS (xylazine-sevoflurane), XI (xylazine-isoflurane), DS (detomidine-sevoflurane) and DI (detomidine-isoflurane). All horses were fasted for 12 h before premedication; water intake was not restricted. Before premedication, the body weight, respiratory rate, body temperature, and heart rate of the horses were recorded.

Before premedication, a 20-gauge intravenous catheter was placed in the right jugular vein for blood sample collection and administration of anaesthetic drugs. Electrocardiography (ECG) leads (aVL, aVR, aVF) and oxyhaemoglobin saturation (SpO2) were monitored by a BM3 Vet monitor (Bionet, Seoul, Korea) before premedication and during anaesthesia. Intravenous xylazine (1.1 mg/kg b.w., Rompun 2%, Bayer, Mississauga, Canada) was used for premedication in XS and XI groups, and intravenous detomidine (6 µg/kg b.w., Domosedan, Pfizer, New York, NY, USA) was used for premedication in DS and DI groups. Ketamine hydrochloride (2 mg/kg b.w., Ketasol 10%, Richterpharma, Wels, Austria) and midazolam (0.03 mg/kg b.w., Demizolam, Curamed Pharma GmbH. Karlsruhe Germany) were mixed in the same syringe and intravenously administered to induce anesthesia for assisted fall. After induction of anaesthesia, horses were placed in left lateral recumbency and the trachea was intubated with a cuffed endotracheal tube with an internal diameter of 30 mm. The endotracheal tube was attached to a large animal circle breathing system anaesthesia machine (Large animal LSD 3000 anaesthetic machine, Dublin, OH, USA, 12 breath/min), and anaesthesia was maintained with either sevoflurane (Sevorane liquid, Abbott Laboratories, Abbott Park, IL, USA) or isoflurane (Aerrane, Baxter Healthcare, Deerfield, IL,USA) for 60 min. The initial concentration of

sevoflurane was 8% + 4 l O_2/min and was reduced to 4%. After 30 min of anaesthesia, the sevoflurane dose was changed to 2% + 4 l O_2/min, which was maintained until the end of anaesthesia (60 min). The initial dosage of isoflurane was 5% + 4 l O_2/min, which was reduced to 4% after 15 min. At 30 min of anaesthesia, the isoflurane dose was changed to 2.5 % + 4 l O_2/min, which was maintained until the end of anaesthesia (60 min). After 60 min, the administration of inhalation anaesthetics was discontinued, and all animals were supported with O_2 for 10 min.

Clinical evaluations

Before pre-anaesthetic administration and during anesthesia (5th, 15th, 30th and 60th min) heart rate (HR), ECG, body temperature (BT), SPO_2, respiratory rate (RR), systolic, diastolic and mean arterial pressure were measured and recorded.

After initiation of inhalation anaesthesia, palpebral reflex was controlled during and at the end of anesthesia in all groups. Palpebral reflex (sluggish) time (PRs), corneal reflex (strong) (PRS), palpebral reflex (normal) (PRn), spontaneous respiration return time (SRRT) and standing up time (SUT) were recorded on examination papers.

Haematologic evaluations

Before (control) and during anaesthesia (5th, 15th, 30th and 60th min) a 15-ml blood sample was collected from the right jugular vein for hematologic, blood gas, and biochemical evaluations. Red blood count (RBC), white blood count (WBC), blood pH, venous partial carbon dioxide pressure ($PvCO_2$), venous partial oxygen pressure (PvO_2), total carbon dioxide (tCO_2), hemoglobin level (Hb), hematocrit (Ht), bicarbonate (HCO_3-), electrolytes (Na^+, K^+) and lactic acid were measured using a Gem Premier 3000 (Biomerieux Diagnostics, Marcy l'Etoile, France) and a Medonic CA 530 hematology analyzer (PZ Cormay, Łomianki, Poland).

Biochemical evaluations

The serum levels of total protein (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), α-glutamyl transferase (γGT), total bilirubin (T-bil) and glucose (Glu) were measured before and at the end of anaesthesia (60th min) using a VetTest 8008 biochemical analyzer (Idexx Laboratories, Westbrook, ME, USA).

Intraocular pressures measurements

Before pre-anaesthetic administration, eyes examination (palpebral, corneal and pupillary reflexes, conjunctiva) were done in all horses and intraocular pressures were measured using a TonoVet tonometer (Icare, Vantaa, Finland) before administering xylazine and detomidine (control) and at the 5th, 15th, 30th, and 60th min after inducing anaesthesia. The same eyes (right) were evaluated in all horses.

Data and statistical analysis

IBM SPSS 21 statistics programs were used to analyze the data. The Shapiro-Wilk test was used for normality then the one and two-way repeated measure (ANOVA) followed Tukey significance tests to compare intra-group and inter-group values. The level of statistical significance was set at $P < 0.05$. Results were presented as Mean ± SE (Standard Error).

RESULTS

Clinical evaluation results

In the current study, suitable and adequate sedation was provided in all groups. The clinical evaluations are shown in Table 1. There were significant differences seen in PRsT and SUT values between groups. The PRsT values of sevoflurane groups were shorter than those of the isoflurane groups ($P < 0.05$). The SUT values were higher in group DS than in the others ($P < 0.05$).

The cardiopulmonary findings are shown in Table 2. Increases and decreases were seen in HR, SpO_2

Table 1. Clinical Evaluation of groups (Mean ± SE) PRsT; Palpebral reflex sluggish time, PRST; palpebral reflex strong time, SRRT; spontaneous respiration return time, SUT; standing up time, a,b,c; P < 0,05 between groups.

Group	PRsT	PRST	SRRT	SUT
XS	5.98±0.57[a]	14.28±3.31	10.42±4.47	39.83±8.63[a]
XI	17.66±2.61[b]	14.54±0.80	18.55±1.21	32.00±4.41[a]
DS	5.80±0.71[a,c]	17.23±1.05	9.81±3.83	79.50±14.39[b]
DI	18.00±4.06[b]	14.50±3.13	23.66±6.29	26.83±1.72[a]

Table 2. Cardiopulmonary evaluation of groups (M±SE)

Values	Groups	0th	5 th	15th	30th	60th	Reference ranges
HR	XS	57±8.42	47.6±12.04	62.8±13.33	52.1±10.99	51.3±9.24	28-45
	XI	54±8.42	68±12.04	55.8±13.33	55±10.09	55.5±9.24	28-45
	DS	46.8±8.42	51.6±12.04	56±13.33	34.8±10.99	31.8±9.24	28-45
	DI	34.3±8.42	35.6±12.04	49.8±13.33	60.5±10.99	54.3±9.24	28-45
SpO_2 %	XS	92.3±1.77	95.5±0.77	96.5±0.62	95.3±1.30	95±1.34	85-100
	XI	95.6±1.37	97.8±0.77	97.1±0.62	94.6±1.30	94.6±1.34	85-100
	DS	96.5±1.77	96.5±0.77	96.1±0.62	95.1±1.30	94.1±1.38	85-100
	DI	94.3±1.77	96±0.77	96.5±0.62	95.5±1.30	96±1.34	85-100
RR	XS	15.5±4.01	16.6±4.54	19.4±5.74	14.4±3.17	11.4±1.43	8-20
	XI	13.1±2.61^{ab}	27.3±9.38^{a}	11.6±2.04^{ab}	7.2±1.20^{b}	7.6±0.98^{b}	8-20
	DS	13.1±2.92	9±1.26	10.8±1.55	11.5±1.38	12.8±2.70	8-20
	DI	10.4±2.56	8.75±2.78	13.5±4.99	18±7.18	7.6±1.60	8-20
BT	XS	36.8±0.09^{a}	36.7±0.08^{a}	35.9±0.12^{b}	35.7±0.18^{b}	35.7±0.2^{b}	37.5-38
	XI	37.1±0.21	37±0.22	36.5±0.42	36.3±0.41	36.1±0.30	37.5-38
	DS	37±0.26	36.6±0.31	36.5±0.46	36.5±0.40	36.3±0.37	37.5-38
	DI	37.1±0.34	36.8±0.34	37.1±0.27	36.9±0.35	36.9±0.40	37.5-38
SP	XS	153.3±7.81	105.3±5.57	151.5±10.14	107.8±10.17	87.8±6.22	112±14
	XI	161.8±7.8	158.5±5.57	125.6±10.14	118.3±10.17	103.3±6.22	112±14
	DS	153.6±7.81	153.6±5.57	122.5±10.14	118.3±10.17	103.3±6.22	112±14
	DI	160.1±7.81	155.5±5.57	122.8±10.14	106.6±10.17	94.6±6.22	112±14
DP	XS	59.6±10.64^{*,a}	58.3±8.57^{*,a}	121.6±7.3^{*,b}	104.1±5.82^{*,b}	70.8±7.50^{a}	70±14
	XI	117±10.64^{a}	107±8.57^{*,a}	80.3±7.36^{a}	61.5±5.82^{b}	47±7.50^{b}	70±14
	DS	119.3±10.64^{a}	83±8.57^{*,b,c}	80.3±7.36^{a,c}	61.5±5.82^{b,c}	56.6±7.50^{b,c}	70±14
	DI	124.1±10.64^{a}	95.5±8.57^{*,b}	80.3±7.36^{b}	61.5±5.82^{b}	60.6±7.50^{b}	70±14
MP	XS	99.5±19.86	77±12.5	132.8±8.14	106.1±6.57*	80.3±4.31	90±14
	XI	109.4±21.76^{a,c}	114.4±13.73^{a}	97.2±8.92^{a}	74.6±7.20^{b,c}	68.6±4.72^{b,c}	90±14
	DS	98.6±19.86^{a}	111.5±12.54^{a}	91.6±8.14^{a}	72.8±6.57^{b}	67.1±4.31^{b}	90±14
	DI	96±19.80^{a}	111.5±12.50^{a}	91.6±8.12^{a}	72.8±6.55^{b}	67.1±4.28^{b}	90±14

HR; heart rate, SpO_2; oxygen saturation, RR; respiratory rate, BT; body temperature, SP; systolic pressure, DP; diastolic pressure, MP; mean pressure, *,**; $P < 0.05$ between groups evaluation, a,b,c ; $P < 0.05$ in groups evaluations. References: Arıcan et al. 2015.

and SP (systolic pressure) values before and during anaesthesia in all groups. The changes detected in these values were not statistically significant. Respiratory rate (RR) increased at 5 min and decreased from 15 min until the end of anaesthesia in XI ($P < 0.05$). Significant differences were seen in diastolic (DP) and mean pressures (MP) between and within groups ($P < 0.05$). Decreases of DP were recorded at 5th, 15th, 30th, 60th min of anaesthesia in XI, DS and DI. Increases of DP were detected at 15,

Table 3. Hematological evaluation of groups (Mean ± SE).

30, 60 min of anaesthesia in XS. In XI, DS and DI the MP increased at 5 min of anaesthesia and decreased until the end of anesthesia. In XS the MP decreased at 5 min and increased at 30 min of anaesthesia. BT decreased from 15 min until the end of anaesthesia in XS. The decrease of BT was statistically significant ($P < 0.05$).

Haematologic results

The measured hematologic and blood gas parameters are shown in Table 3. In hematologic evaluations, RBC, WBC, Hb, Ht and lactic acid values showed changes. The changes of RBC, WBC, Hb and Ht values were in reference intervals in all groups. The lactic acid levels were lower than reference range before and during anaesthesia in all groups. In DS, there was significant difference recorded in lactic acid levels between 0th min and the other times.

The pH values decreased at 15th min of anaesthesia in DS. This reduction was below the reference range and it was significant ($P < 0.05$) (Table 3). At 0th, 5th, 15th, 30th, 60th min of anaesthesia, the $PvCO_2$ values were higher in sevoflurane groups (XS and DS) than in isoflurane groups (XI and DI). The $PvCO_2$ values increased during anaesthesia in sevoflurane groups. After 5 min of anaesthesia, the $PvCO_2$ values started to decrease in isoflurane groups, and the reductions were significantly different ($P < 0.05$). There were no significant differences seen in PvO_2 values between groups at the 0th, 5th, 15th, 30th and 60th min of anaesthesia. Before and during anaesthesia the PvO_2 values were lower than reference range in DI ($P < 0.05$) (Table 3). Differences in tCO_2 values were seen in all groups. The tCO_2 values were higher than reference range at 15th, 30th and 60th min of anaesthesia in XS and DS ($P < 0.05$). The other differences were in reference interval (Table 3). The HCO_3 levels of XS and DS were significantly different from XI and DI ($P < 0.05$).

Biochemical results

Biochemical parameters are shown in Table 4. There were significant differences detected in TP, AST, ALT and ALP values among and within groups. All of the values were within the normal range (Table 4). T-bil values were higher at 0 and 60th min of anesthesia than the reference range in XS. At the end of the anaesthesia Glu values in XS, XI and DI were higher than XS and reference values (Table 4).

Intraocular pressures results

Intraocular pressures (IOP) were measured before and during anesthesia (5th, 15th, 30th and 60th min) (Table 5). There were no significant differences observed between and within groups (reference range: 13-37 mmHg).

DISCUSSION

Arıcan et al. (2015) compared the clinical effects of sevoflurane, isoflurane, and medetomidine anaesthetic combinations in horses. They found the loss of palpebral reflex, return of palpebral reflex and standing up times to be shorter in a sevoflurane group than in an isoflurane group. They attribute this to the low blood concentration of sevoflurane at a plane of anaesthesia and its relatively rapid elimination from the body. In our study, there were significant differences observed in PRsT and SUT ($P < 0.05$). Palpebral reflex sluggish time of sevoflurane groups was shorter than those of isoflurane groups which may be supported by the previous finding of low blood concentration and rapid elimination of sevoflurane from the blood. Detomidine binds α-2 adrenoreceptors in the locus coeruleus and medulla spinalis to provide sedative and analgesic effects. It suppresses norepinephrine and dopamine release in CNS. Because of this, detomidine is more potent and has fewer side effects than xylazine. It has been reported that detomidine produces deeper and longer sedation than xylazine (Steffey and Pascoe, 2002; Rochbrach et al., 2009). In this study, SUT was longer in XS and DS than XI and DI. The longest and shortest times were detected in DS and DI. This condition suggested that anaesthetic combinations, anaesthetic doses and animal related factors (age, sex and weight) could influence the post anaesthetic parameters.

Generally cardiac arrhythmias which occur during anaesthesia can cause serious problems, and

Values	Groups	0th	5th	15th	30th	60th	Reference ranges
RBC (x10^6/mm^3)	XS	9.8±0.12	8.8±0.63	8.6±0.81	7.7±0.53	7.9±0.48	7-13
	XI	10.3±0.43	8.1 ±0.34	7.5±0.28	7.3±0.33	7±0.19	7-13
	DS	10.6±0.38	10.8±0.28	10.7±0.18	10.1±0.32	9.3±0.37	7-13
	DI	9.7±1.07	10.9±0.87	11.4±0.48	11±0.32	10.6±0.43	7-13
WBC (x10^3/mm^3)	XS	9±1.06	8.34±0.93	7.64±0.84	8±1.01	6.96±0.75	6-12.5
	XI	10.6±0.74	9.4±0.63	9.2±0.59	8.6±0.66	8.3±0.63	6-12.5
	DS	7.8±0.42	8.9±1.23	8.6±1.42	8.65±0.95	7,6±0.60	6.12.5
	DI	8±1.20	9.1±1.26	9.2±1.07	9.6±0.95	9.5±0.95	6-12.5
pH	XS	7.3±0.02	7.3±0.02	7.3±0.02	7.3±0.02	7.3±0.02	7.36-7.43
	XI	7.4±0.001	7.3±0,001	7.4±0.01*	7.4±0.08	7.4±0.03	7.36-7.43
	DS	7.4±0.001^{a}	7.3±0.02^{ab}	7.2±0.02^{*,b}	7.2±0.02^{b}	7.2±0.02^{b}	7.36-7.43
	DI	7.4±0.01	7.3±0.02	7.3±0.01	7.4±0.03	7.4±0.01	7.36-7.43
$PvCO_2$ (mm/Hg)	XS	47.2±1.06^{*,c}	52.6±1.6^{*,bc}	55.8±1.74^{*,b}	59.2±1.24^{*,ab}	63.4±1.99^{*,a}	38-48
	XI	36.9±1.44**	38.2±1.33**	32.9±0.95**	29.6±4.60**	33.3±2.19**	38-48
	DS	45.4±0.92^{*,c}	52.8±3.15^{*,bc}	59.4±3.77^{*,abc}	64.5±5.43^{*,ab}	70.2±5.89^{*,a}	38-48
	DI	36±0.45^{**,ab}	38.6±1.87^{**,a}	36.4±0.88^{**,ab}	33.8±1.30^{**,ab}	32.8±1.52^{**,b}	38-48
PvO_2 (mm/Hg)	XS	30.6±1.32	42.8±2.92	48.8±8.5	52.6±7.28	48.2±8.16	37-56
	XI	29.6±1.09^{c}	37.5±1.72^{bc}	42.4±1.79^{ab}	43.9±3.21^{ab}	48.7±2.3^{a}	37-56
	DS	31.2±2.03^{bc}	28.8±1.35^{c}	42.8±1.80^{ab}	47.5±4.17^{a}	55.2±6.2^{a}	37-56
	DI	33.5±3.19^{ab}	29.2±1.56^{b}	33.1±0.90^{ab}	34.3±1.56^{ab}	31.6±2.0^{b}	37-56
tCO_2	XS	32.9±0.73*	33.8±1.37	34.1±1.49*,**	35.9±1.04	37.3±0.95*	24-32
	XI	27.2±0.83*	26.5±1.20	25.1±1.10*	21.1±4.31	26.6±0.77**	24-32
	DS	33.4±0.73^{**b}	33.2±1.16^{b}	34.1±1.08^{*,**,ab}	35.9±1.28^{ab}	39.2±2^{*,a}	24-32
	DI	25.9±0.59*	26±1.48	25.4±0.83	24.8±0.54	25.1±1.07**	24-32
Hb (g/dL)	XS	14.6±0.30	13.22±1.03	12.56±1.02	11.66±0.94	11.8±0.83	11-19
	XI	15.5±0.55^{a}	12.3±0.51^{b}	11.5±0.41^{b}	11±0.46^{b}	10.7±0.2^{b}	11-19
	DS	15.3±0.27	15.4±0.51	15.3±0.54	14.4±0.70	13.4±0.39	11-19
	DI	13.5±1.18	15.8±0.63	16.2±0.53	15.4±0.36	14.8±0.33	11-19
Ht (%)	XS	50.22±1.60	45.16±3.42	44±3.66	39.74±3.21	40.2±2.60	35-52
	XI	52.3±1.93	40.5±1.66	37.8±1.39	36.3±1.57	34.8±0.75	35-52
	DS	54.8±1.87	55.94±1.88	55.3±1.71	51.4±3.05	47.6±2.30	35-52
	DI	44.6±4.61	51.4±2.50	53.6±1	53.1±1.51	51.1±1.40	35-52
HCO_3 (mm/L)	XS	31.4±0.73*	32.1±1.37	32.4±1.46*,**	34.1±1.04*	35.4±0.94*	22-29
	XI	26±0.79**	25.4±1.18	24±1.08*	24.3±1.12**	25.5±0.74**	22-29
	DS	32±0.70^{*,ab}	31.6±1.07^{b}	32.3±1^{**,ab}	33.9±1.10^{*,ab}	37±1.83^{*,b}	22-29
	DI	24.8±0.59**	24.8±1.43	24.3±0.83*	23.8±0.54**	24±1.04**	22-29
Na (mEq/L)	XS	136.4±0.87	136±0.54	137±1.04	136.2±1.06	135.2±0.96	136-142
	XI	142.8±0.30	142±1.26	142.6±1.35	127.4±16.46	140.6±1.99	136-142
	DS	138±0.83	138.6±0.87	137.2±0.96	137.7±1.25	136.5±0.47	136-142
	DI	140±1.06	138±2.25	135±1.92	137±1.44	137.6±1.08	136-142
K (mEq/L)	XS	3.5±0.17	3.3±0.20	3.4±0.26	3.6±0.30	3.7±0.28	2.2-4.6
	XI	2.9±0.06	2.8±0.11	2.7±0.11	26.4±23.27	2.8±0.16	2.2-4.6
	DS	3.5±0.19	3.8±0.23	3.4±0.23	3.7±0.29	3.8±0.36	2.2-4.6
	DI	3.2±0.07	3.5±0.09	3.4±0.05	3.4±0.05	3.5±0.08	2.2-4.6
Lactic acid (mg/dL)	XS	0.6±0.12	1.1±0.26	1±0.20	0.7±0.04	1±0.16	4-12
	XI	0.6±0.25	1.1±0.30	1±0.18	08±0.05	1±0.15	4-12
	DS	0.5±0.05^{b}	1.6±0.10^{a}	1.6±0.15^{a}	1.5±0.20^{a}	1.2±0.50^{a}	4-12
	DI	0.6±0.25	1.1±0.35	1±0.15	0.8±0.15	1±0.15	4-12

RBC; red blood count, WBC; white blood count, PH; blood PvH, $PvCO_2$; venous partial carbon dioxide, PvO_2; venous partial oxygen pressure, tCO_2; total carbon dioxide, Hb; hemoglobin level, Ht; hematocrit, HCO_3; bicarbonate, *,**; P < 0.05 between groups evaluation, a,b,c; P < 0.05 in groups evaluations. References: Kawamura 2011, Aros et al. 2017, http://cal.vet.upenn.edu/projects/fieldservice/Equine/EQCLPATH.htm

Table 4. Biochemical evaluations of groups (Mean ± SE)

Values	Groups	0th	60th	Reference ranges
TP (g/dL)	XS	7.4±0.27	6.9±0.18	5.6-7.9
	XI	7.05±0.18 [a]	6.2±0.10 [b]	5.6-7.9
	DS	7.07±0.25 [a]	6.47±0.26 [b]	5.6-7.9
	DI	7.4±0.25	6.9±0.20	5.6-7.9
AST (g/dL)	XS	337.66±30.98	320.16±34.3	100-600
	XI	591±213.50	345.25±160.08	100-600
	DS	410.75±171.55	356.25±146.08	100-600
	DI	450±52.23	345±122.05	100-600
ALT (IU/L)	XS	25±3.52	28±5.97	5-50
	XI	28±8.71	40.75±9.97	5-50
	DS	18.50±6.38	30.50±10.41	5-50
	DI	25.2±7.28	42.3±6.65	5-50
ALP (IU/L)	XS	3.2±0.20	2.86±0.12	1.9-3.2
	XI	3.07±0.23	2.65±0.11	1.9-3.2
	DS	3.40±0.12 [a]	2.87±0.08 [b]	1.9-3.2
	DI	3.05±0.23	2.58±0.15	1.9-3.2
T-bil (mg/dl)	XS	4.96±0.98	4.26±0.65	0-3.5
	XI	4.15±0.21	3.40±0.44	0-3.5
	DS	3.40±0.83	2.95±0.68	0-3.5
	DI	4.1±0.65	3.2±0.78	0-3.5
γGT (U/L)	XS	15±2.8	15±2.7	0-87
	XI	11.25±3.03	18.25±8.08	0-87
	DS	14.75±2.46	12.50±3.17	0-87
	DI	16±3.05	19±2.18	0-87
Glu (IU/L)	XS	105.16±7.53	169.66±2.80	64-150
	XI	92.25±1.49[a]	130.50±5.7[b]	64-150
	DS	88±2.16[a]	186.75±17.01[b]	64-150
	DI	104.2±8.57	165.6±3.33	64-150

TP; total protein, AST; aspartate transaminase, ALT; alanine transaminase, ALP; alkaline phosphatase, T-bil; total bilirubin, γGT; α-glutamyl transferase, Glu; glucose, a,b,c; P < 0.05 in groups evaluations. References: Kawamura 2011, Takasu et al., 2013, Aros et al., 2017, http://cal.vet.upenn.edu/projects/fieldservice/Equine/EQCLPATH.htm

monitoring is required (Muir and Hubbell, 2009; Erol and Arıcan, 2017). Anaesthetic agents cause dose-dependent reductions in arterial blood pressure with myocardial depression and peripheral vasodilatation. Many investigators (Ebert et al., 1995; Aida et al., 1996; Röding et al., 1996; Duke-Novakovski et al., 2015) have reported this dose-dependent suppression of the cardiovascular system. Sevoflurane and isoflurane have been reported to be equally arrhythmogenic in animals (Matthews and Hartsfield, 2004). In this study HR, SpO_2, BT, RR, SP, DP and MP values were recorded at the beginning of the anaesthesia and SP and MP values were higher than the reference ranges in all groups (Table 2). In this study HR, SpO_2, BT, RR, SP, DP and MP values were recorded at the beginning and during of the anaesthesia. DP and MP values showed significant decreases and increases during anesthesia in all groups (P < 0.05). DP values were higher than reference range at 0th 5th and 15th min of anaesthesia in XI, DS and DI. The statistically differences were detected between groups at 0th, 5th and 30th min of anaesthesia in DP values. Intra-groups evaluation of DP values, there were statistically differences recorded at 0th and other times in all groups. In XS, MP value was statistically different at 30th min of anaesthesia than others. Intra-groups evaluation of MP values, the statistically differences were detected between 0th, 5th, 15th and 30th, 60th min of anaesthesia in XI, DS and DI.

However, evaluations of ECG traces from all groups did not show atrioventricular blockade, atrial fibrillation, hypoxia-related extra systoles or dysrhythmias. The electrolyte parameters correlated well with our ECG evaluations as there were no significant differences detected in Na and K values.

Taylor and Clerk (2007) reported that reduction of BT can occur secondary to variations in skin thickness and environmental temperature in horses. In our study, BT showed decreases during anaesthesia in all groups. However, the decrease was significantly different only in XS.

The horse's hematologic profile is affected by different factors which are nutritional, environmental, age, sex, performance, and genetics (Padalino et al., 2014). At the same time stress, excitement, fear and the associated catecholamine exchange in blood circulation, as well as

Table 5. IOP evaluation of groups (Mean ± SE)

	Groups	0th	5th	15th	30th	60th	Reference range
IOP (mm/Hg)	XS	24±0.6	27.8±3.81	38.7±2.95	34.7±2.58	35.4±1.15	13-37
	XI	32±1	34.2±0.50	34.2±1.15	33.9±0.57	34.4±0.50	13-37
	DS	52.3±4.45	32.7±3.73	50.3±2.71	42.3±2.59	41.5±3.33	13-37
	DI	34.4±2.58	25.7±1.78	20.2±0.34	21.1±0.22	20.1±0.48	13-37

IOP; intraocular pressure, Reference; Komáromy et al., 2006; Monk et al., 2017

hyperglycemia and hypoxia can all cause changes in venous blood parameters (Robinson, 2009). These are potential reasons for the changes of RBC, WBC, Hb, Ht and lactic acid at the beginning of and during anaesthesia in the present study.

Analysis of blood gas samples from arterial and venous blood give information about ventilation and fluid-electrolyte balance (Hartsfield et al., 2006; Erol and Arıcan, 2017).In venous blood gas parameters, the significant differences were detected in pH, $PvCO_2$, PvO_2, tCO_2 and HCO_3 values ($P < 0.05$). The changes in pH values were noted during anesthesia in all groups. Depending on the changes of pH values, effected in $PvCO_2$, PvO_2, tCO_2 and HCO_3- values. These changes were more evident in sevoflurane groups and related to the compensatory response to respiratory acidosis and metabolic alkalosis. Venous blood values (pH, $PvCO_2$, PvO_2, tCO_2 and HCO_3) are 5-10 mmHg and 1-5 mEq/l higher than arterial values (Steffey et al., 2005; Marcilla et al., 2012).

The α-2 agonists cause cardiac depression and hypoxemia. The resulting cardiac depression and hypoxemia decrease PO_2 and increase PCO_2 values. In addition, inhalation anaesthetic agents cause respiratory depression and increase in PCO2 values. The level of depression depends on doses of inhalation agents (Kronen, 2003). Detomidine is 10 times more potent and produces deeper and longer sedation than xylazine (Yamashita et al., 2000; Steffey et al., 2005; Padalino et al., 2014). The effects of sevoflurane on CNS are lower than isoflurane and compared to other inhalation anesthetics the suppression effect is greater at increasing doses. Sevoflurane reduces systemic vascular resistance, arterial blood pressure and mean pulmonary arterial pressure during anaesthesia. However, these conditions are not fully observed during anesthesia and may vary depending on the anaesthetic duration (Mutoh et al., 1997; Muir and Hubbell, 2009). The decreases of anaesthetic agent doses during the anaesthetic period and the concurrent ECG and venous blood parameters evaluations support the literature findings (Mutoh et al., 1997; Kronen, 2003; Padalino et al., 2014). Yamashita et al. (2006) emphasized that α-2 agonists and ketamine-midazolam combinations cause to decrease PO_2 values but do not significantly change PCO_2 and cardiovascular findings. They have also been associated with intrapulmonary shunts and ventilation-perfusion mismatch. Intermittent positive pressure ventilation (IPPV) suppresses the cardiovascular functions during general anaesthesia in horses because the mechanical ventilation increases thoracic pressure and retards venous blood circulation (Taylor and Clerk, 2007). Kushiro et al. (2005) reported that cardiovascular functions are more suppressed than during normal spontaneous breathing during anesthesia in horses due to the increased amount of catecholamine in circulation. There were statistically differences recorded in $PvCO_2$, PvO_2, tCO_2 and HCO_3 values between and among groups. In XS and DS, $PvCO_2$ values were higher than XI and DI at 5, 15, 30 and 60th min of anesthesia. PvO_2, tCO_2 and HCO_3 values of DI were lower than the others at same times. The highest values of $PvCO_2$, PvO_2, tCO_2 and HCO_3 were detected in DS at 60th min of anesthesia. In the current study, the observed decreases and increases in $PvCO_2$, PvO_2, tCO_2 and HCO_3 values in groups were thought to be due to the changes in the amount of circulating catecholamine and ventilation support, and varied

depending on the anesthetic agents' doses.

The biochemical values may be different in the pre-anaesthetic, anaesthetic and post-anaesthetic periods due to differences in anaesthetic agents. It is important to have information about liver function before and after anaesthesia. The differences in these values because of detoxification of anaesthetic agents in liver balance the anaesthetic agents' effects on liver (Steffey, 2009; Hubbell et al., 2011). In the present study, the biochemical parameters' levels changed after anaesthesia. However, the TP, AST, ALT and ALP values detected, although significantly different, were still within the reference interval (Table 4). T-bil values were higher at 0 and 60th min of anesthesia than reference range in XS. At the end of anaesthesia Glu values in XS, DS and DI were higher than in XI and the reference values. The use of α-2 agonists in horses cause to decrease in TP value and hyperglycaemia (Muir and Hubbell, 2009). In our study TP values decreased in all groups at 60th min. This condition supported the literature information. The changes of AST, ALT and ALP values between 0th and 60th min in all groups were thought due to detoxification of anaesthetic agents in liver. The significant difference of ALP value in DS was more evident than others. However, it was within reference interval. The higher T-bil values reason was thought to be due to age, sex and gender of horses. The increases of Glu values in all groups supported the anaesthetic agents' effects on liver.

Ketamine is the most commonly used dissociative anaesthetic agent in horses. It has been reported that it increases renal blood flow, mucous secretions of the respiratory tract and the amount of catecholamine in circulation. The fact that ketamine is metabolized in the liver was reported in previous studies (Hall et al., 2000; Rosetti et al., 2008; Levionnois et al., 2010; Oku et al., 2011). Liver function during anaesthesia depends on liver blood flow, metabolites of anaesthetic agents and other drugs. Isoflurane and sevoflurane are less hepatotoxic than other inhalation anaesthetics (Topal et al., 2003). Our study supported the literature's previously reported information (Hall et al., 2000; Topal et al., 2003; Rosetti et al., 2008; Levionnois et al., 2010; Oku et al., 2011).

In general anaesthesia, anaerobic metabolism begins to produce lactate due to decreased perfusion in tissues and an increase in the amount of lactate during anaesthesia. Along with anaerobic metabolism Glu levels increase due to sympathetic stimulation and lipolysis (Edner et al., 2005). Steffey and Pascoe (2002) emphasized that α-2 agonists increase the serum Glu levels in a dose-dependent manner. In XS, DS and DI the Glu values were greater than reference limits at the end of the anesthesia (Table 4). The increase of Glu values were significant in XI and DS. However, DS Glu values were higher than reference interval. The changes of Lac values were detected in groups but they were in reference limits. The measured Glu values in our study showed changes due to anaerobic metabolism during the anaesthesia.

The IOP measurement is part of the routine eye examination in horses with tonometry and has become more widely used in recent years. The reference range for horses using tonometry is reported as 15-37 mmHg (Komáromy et al., 2006; Monk et al., 2017). Several factors such as the time of day, sedation, anesthetic agents and head position influence the IOP (Hall et al., 2000; Stine et al., 2014; Arıcan et al., 2015; Monk et al., 2017). In the present study, there were no statistical differences seen in IOP values at the 0th, 5th, 15th, 30th and 60th minutes of anaesthesia between and within groups. The IOP values of DS were higher at the 0th (Control), 15th, 30th and 60th min of anesthesia. No statistical differences were detected between and within groups. These differences were thought to be related to the anaesthetic agents' effects on the intracranial and cerebral perfusion pressures. On the other hand, these results suggest that the anaesthetic combinations used in our study can be used safely for eye surgeries in horses.

CONCLUSION

In conclusion, this study shows that the anaesthetic combinations of sevoflurane and isoflurane with xylazine and detomidine provided safe and suitable anaesthesia in horses. It is a comprehensive study on the evaluation of anaesthesia combinations in horses and the transfer of our experience to clinicians. There were statistical differences

in hematologic, biochemical and cardiovascular parameters even when these values were found within normal physiological limits. It was determined that both inhalation anaesthetic drugs studied could depress the respiratory system due to dose-dependent effects, and similar effects were observed between sevoflurane and isoflurane anaesthesia on the respiratory and circulatory systems. It has been clinically determined that the choosing of α-2 agonist and their action times are very important in general anaesthesia. The cardiopulmonary changes were more prominent in sevoflurane groups. Our study did not reveal any statistical differences in IOP measurements. However, it should be noted that intraocular pressures were measured with the animals lying down and our results do not rule out changes in IOP in a standing position. We concluded that these anaesthesia protocols are suitable for ophthalmic surgery.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effects of Rapid and Ultra-Rapid Fluid Resuscitation Guided by Blood Lactate Clearance Rate in Diarrheic Calves

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ABSTRACT. The assessment of the results of intravenous fluid treatment in diarrheal calves is difficult under field conditions. To determine the effects of ultra-rapid (1 h) and rapid (3 h) intravenous infusions with 0.9% NaCl and 1.3% NaHCO3 solutions at a dose of 60 ml/kg on lactate clearance (LC) in calves with diarrhea. Sixty calves, including a healthy control group (n=20) and a group of calves with diarrhea (n=40), were used. Diarrheic calves were divided into two groups (n=20) according to solution type, and each group was then divided into two equal subgroups according to the infusion rate (n=10). Clinical and laboratory inspections of diarrheic calves were performed pre- and post-infusion, and the healthy control group was examined once. LC rates were calculated in the 1 h and 3 h subgroups. Marked improvements in clinical findings related to dehydration were observed in all groups with neonatal diarrhea that were given intravenous infusions with 0.9% NaCl and 1.3% NaHCO3 solutions. End of the infusion, the LC of the 0.9% NaCl and 1.3% NaHCO3 solutions in the ultra-rapid groups was significantly increased by 36.4% and 31.8%, respectively. However, for rapid infusion of the same solutions, 13.6% and 31.8% increases were observed, but the differences were not significant. Under field conditions, the LC of L- and D-lactate varied with the infusion rate, and these variations were significant in ultra-rapid subgroups for both solutions. Further studies will be designed for fluid therapy in calves based on the calculation of LC.

Keywords: Calf diarrhea, blood lactate clearance, rapid infusion, sodium bicarbonate, sodium chloride.

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INTRODUCTION

Major challenges in calf husbandry tend to occur in the neonatal period (0-28 day), and the primary problem during this period is calf diarrhea (Başoğlu et al., 2004; Sen and Constable, 2013). The high level of morbidity and mortality in the neonatal period is associated with complex etiological factors as well as the inability to perform effective treatments and the radical application of fluid therapy (Abeysekara et al., 2007). Metabolic acidosis and dehydration are other common problems that require specific treatments. Under field conditions, dehydration and metabolic acidosis of calves with diarrhea are estimated based on clinical signs due to the lack of laboratory equipment (Nakagawa et al., 2007). Metabolic acidosis occurs through the loss of bicarbonate through feces, which reduces the excretion of H ions from the kidneys and the accumulation of organic acids, such as lactate in the blood (Lorenz, 2009). Several studies have been published on lactate and D-lactatemia in calves with or without diarrhea (Omole et al., 2001; Ewaschuk et al., 2003), whereas the literature published on lactate in humans has focused on biomarker properties in sepsis and the assessment of trauma situations (Gomez and Kellum, 2015; Lee and An, 2016; Dekker et al., 2017).

The main goal of intravenous (IV) fluid therapy in diarrheic calves is to replace fluids, electrolytes, buffer and energy deficits. The route, amount, type and the rate of application of IV fluids for calves can change or are not suitable under field conditions (Kaske, 1994; Berchtold, 1999). Berchtold (1999) also stated that rapid infusion may cause cerebral edema, pseudoanemia, and hypoproteinemia. However, Kasari and Naylor (1985) as well as Kasari (1990) and Constable (2003) have recommended isotonic crystalloids for dehydrated calves at a maximum dose of 80 ml/kg/hr by an intravenous route. Another study found that an infusion rate of 80 ml/kg/hr may lead to hypernatremia and recommended that the dose be as low as 30 or 40 ml/kg/hr (Grove-White, 2007). Nevertheless, in children with neonatal diarrhea, Nager and Wang (2010) revealed the favorable effects of 1-h rapid infusions of isotonic crystalloid solutions at a dose of 60 ml/kg.

Lactate clearance (LC) is a simple and inexpensive clinical parameter based on the measurement of lactate levels between two serial times (Jones et al., 2010). LC indicates kinetic alterations in anaerobic metabolism, tissue hypoxia and septic conditions (Nguyen et al., 2010). In recent years, research has focused on this biomarker to discover the effects of fluid therapy in trauma and sepsis patients (James et al., 2011; Yu et al., 2013; Ghneim et al., 2013; Lyu et al., 2015). Interestingly, lactate clearance has not been systematically studied in fluid resuscitation in calves with diarrhea.

In this study, we aimed to determine the efficacy or inadequacy of ultra-rapid (1 h) and rapid (3 h) intravenous infusions with 0.9% NaCl and 1.3% $NaHCO_3$ solutions administered at a dose of 60 ml/kg on lactate clearance in clinically dehydrated neonatal calves with diarrhea.

MATERIALS AND METHODS

Animals and clinical examination

Sixty animals, including healthy (n=20) and diarrheic (n=40) Holstein calves with moderate dehydration from both sexes and 1-3 weeks of age, were enrolled in the study. The clinically healthy control group calves were selected from 4 different dairy cattle farms in Aydin province in the first three-week period of their lives and included in the study. Diarrheic calves in the first three weeks of age that had moderate dehydration were referred to the clinic of researcher and author Erdogan for examination and treatment. Healthy calves were animals that received no treatment or had no infection history, whereas diarrheic calves had a treatment history.

Moderate dehydration (6 - 8%) was determined according to predictors, including skin turgor, dry mucous membranes and eyeball recession of 2 mm (Sen and Constable, 2013). Along with these clinical findings, the diarrheic calves were divided into two major groups (n=20 in each group) for the isotonic 0.9% NaCl solution and 1.3% NaHCO3 solution. The major groups were divided into two subgroups (n=10 in each group) for comparison of the effects of rapid (3 hr) and ultra-rapid (1 hr) infusions. Pre-treatment (Pre) and post-treatment (Post) clinical (e.g. eyeball recession, skin turgor, ability to stand,

suckling reflex, and mucosal membrane moistness) and laboratory data (Lactate, and D-lactate) were recorded.

Infusion protocol and blood samples

Calves in both groups were weighed with an electronic veterinary scale (Vaw 300 4P, Turkey) before treatment. The amounts of fluid for rehydration for both the 0.9% NaCl and 1.3% NaHCO3 solution groups were calculated based on the dosage of 60 ml/kg b.w. Fluid resuscitation was performed for all diarrheic calves in the Vena jugularis intravenously (Braun: 16 G 2" 1.7×50 mm 196 ml/min Lot: 1G16258253, Germany). For each diarrheic calf, the calculated amount of total fluid was infused with an infusion pump (Biocare IP12, Shenzhen Biocare Bio-Medical Equipment Co. Ltd, China). By changing the infusion rate of the pump, the total of both fluids (0.9% NaCl and 1.3% NaHCO3) was infused ultra-rapidly (1 hr) and rapidly (3 hr) to all subgroups. Diarrheic calves were randomly selected for the subgroups of both isotonic crystalloid solutions.

Blood samples were collected in lithium heparin (BD, USA) and a heparinized syringe (Genject Ca2+LH-100 I.U., Turkey) for biochemical and blood gas analyses prior to treatment and post-treatment. Post-treatment blood samples were taken in all infusion groups at the end of the fifth minute after the infusion completed. Heparinized blood samples taken pre- and post-treatment were centrifuged, and the obtained plasma was frozen at -20°C in Eppendorf tubes until analysis. L-lactate concentrations were detected via a hand held portable blood gas analyzer (EPOC, UK) and D-lactate concentrations were determined with a spectrophotometric method and ELİSA using commercial test kits (Biovision, USA).

Lactate clearance

Lactate and D-lactate clearance (percent) were calculated by the following equation described by Nguyen et al.,(2004):

Clearance= "(Lactate pre-treatment)-(Lactate post-treatment)" /"Lactate pre-treatment" X 100

A negative value indicates reduction or clearance of L-lactate or D-lactate, whereas a positive value indicates elevation of lactate or D-lactate pre- and post-treatment intervention.

Statistical analyses

The average (X), standard deviation (SD) and minimum-maximum values (Xmin-Xmax) of the analysis parameters for L-lactate and D-lactate were calculated for each sample interval in the groups. The distribution of numerical data was evaluated using a Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used for comparison of more than two groups in normally distributed parameters, and a Tukey test was used for post hoc comparisons. Nonparametric methods were used for the analysis of non-normally distributed data. A Kruskal-Wallis test with Bonferroni correction was applied for post hoc comparisons for the comparison of parameters in more than two groups. In calves with diarrhea, the T-test and Wilcoxon test were performed on normally and non-normally distributed parameters, respectively, for comparison of pre- and post-treatment values of dependent groups. P values less than 0.05 were accepted as statistically significant ($p<0.05$). SPSS 15.0 software was used to analyze the statistical data.

RESULTS

Calves treated with isotonic NaCl solution at a dose of 60 ml/kg in the ultra-rapid (1 hr) and rapid (3 hr) groups showed marked improvement in skin turgor, mucosal membrane moistness, and eyeball recession, whereas there was no significant improvement in suckling reflexes, which were reduced post-treatment. Seven of ten calves that received ultra-rapid infusion in the 0.9% NaCl group urinated post-treatment; among those treated with rapid infusion, three out of ten calves urinated (30%). In the ultra-rapid infusion (URI) group and rapid infusion (RI) group of 0.9% NaCl, six and five calves were able to stand by themselves, respectively. However, the remaining 9 calves from both 0.9% NaCl solution groups were unable to stand by themselves.

The ultra-rapid infusion subgroup of calves that received the isotonic (1.3%) NaHCO3 solution showed marked improvements in dehydration-related clinical findings, mental status and suckling reflex

post-treatment. In the rapid infusion subgroup of the 1.3% NaHCO3 solution group, these clinical improvements manifested in the second hour of infusion, and the sucking reflex recovered more slowly than in the ultra-rapid infusion group. At the second hour of rapid infusion of 1.3% NaHCO3 solution, all calves urinated; for the ultra-rapid infusion group calves using the same solution, 80% of calves urinated at the end of the infusion. Calves in the 1.3% NaHCO3 solution ultra-rapid infusion subgroup were able to stand by themselves, and the rapid infusion group calves were also able to stand during the second hour of the infusion. Two calves in the 1.3% NaHCO3 URI subgroup had unstable walking. In all infusion groups of calves were rehydrated and the presence and severity of dehydration was clinically reduced.

Table 1. Mean Body weights, amount of solutions and infusion rates in diarrheic calf groups (± s).

Group	*n*	*Body weight (kg)*	*Amount of solution (mL)*	*Infusion rate (mL/min)*
0,9% NaCl Sol. URI	10	29,0 ± 2,5	1742,4 ± 147,8	29 ± 2,4
0,9% NaCl Sol. RI	10	30,0 ± 2,4	1798,2 ± 144,5	10 ± 0,8
1,3% $NaHCO_3$ Sol URI	10	31,4 ± 3,2	1886,4 ± 193,2	31 ± 3,2
1,3% $NaHCO_3$ Sol RI	10	30,2 ± 1,8	1813,2 ± 108,0	10 ± 0,6

There were no significant alterations in the amount of either ultra-rapid or rapid infusions of 0.9% NaCl solution and 1.3% NaHCO3 solution, as shown in Table 1.

L-lactate concentrations were statistically significantly higher for the pre-treatment 0.9% NaCl solution URI, 1.3% NaHCO3 solution URI and 1.3% NaHCO3 solution RI groups than for the control group calves. In the pre-treatment and post-treatment comparisons, ultra-rapid infusion and rapid infusion of both 1.3% NaHCO3 solution and 0.9% NaCl solution resulted in elevations in lactate concentration; these elevations were significant for ultra-rapid infusion of the 1.3% NaHCO3 and 0.9% NaCl solutions. Marked increases in LC were detected in the 0.9% NaCl solution URI, 1.3% NaHCO3 solution URI and 1.3% NaHCO3 solution RI groups. The 0.9% NaCl solution URI and 1.3% NaHCO3 solution URI group lactate clearance rates were 36.4% and 31.8%, respectively, and were statistically significant. There was no significant difference in D-lactate concentrations between the control group and the infusion groups of calves pre-treatment to post-treatment. In the URI and RI groups that received 0.9% NaCl solution, the D-lactate clearance increased, but the D-lactate clearance was reduced in the URI and RI groups that received the 1.3% NaHCO3 solution (Table 2).

DISCUSSION

Research has shown that lactate is a product of anaerobic glycolysis, and the production and clearance of lactate is balanced in body fluids. The daily production of lactate occurs in skeletal muscle, the brain, cardiac muscle, intestines, the renal medulla and erythrocytes; however, it is produced in almost all tissues and organs during disease (Sharkey and Wellman, 2015). The assessment of lactate concentrations has been used as a significant therapeutic and prognostic biomarker for human and animal health for many years (Nguyen et al., 2004; Karagiannis et al., 2006; Figueiredo et al., 2006; Allen and Holm, 2008; Zacher et al., 2010; Tennent-Brown et al., 2010). Lactate is commonly used to determine sepsis and its severity (Gomez and Kellum, 2015; Lee and An, 2016) as well as for the prognostic evaluation of patients with sepsis (Yu et al., 2013; Lyu et al., 2015). For example, in horses with acute abdomen, the influence of perioperative plasma lactate concentrations on the surgical intervention and recovery periods has been shown to be especially important (Donawick et al., 1975; Moore et al., 1976; Genn and Hertsch, 1982), and similar results have been observed in dogs with gastric dilatation and volvulus (De Papp, 1999). The

Table 2. L and D-lactate concentrations and cleareans levels in calves.

Parameter	*Groups*							
	Control	*0,9% NaCl Sol. URI*		*0,9% NaCl Sol. RI*		*1,3% $NaHCO_3$ Sol URI*		*1,3% $NaHCO_3$ Sol RI*	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
L. Lactate (mmol/L)	0,9 ± 0,6	2,2 ± 1,7$^{\sharp a}$	3,0 ± 2,3†^{b}	1,4 ± 1,0	1,4 ± 1,3	4,4 ± 2,8$^{\sharp a}$	5,8 ± 4,2$^{\dagger b}$	2,2 ± 2,1*	2,9 ± 3,2*
D- lactate (mmol/L)	0,4 ± 0,1	0,41 ± 0,02	0,43 ± 0,13	0,39 ± 0,03	0,42 ± 0,14	0,39 ± 0,04	0,38 ± 0,05	0,41 ± 0,05	0,39 ± 0,06
L. Laktat clearens		**36,4%↑**		13,6%↑		**31,8%↑**		31,8%↑	
D- lactate clearens		4,9%		7,7%		- 2,6%↓		- 4,9%↓	

*, †, ♯: Statistically significant from control group p<0,05, p<0,01 and p<0,001, respectively.

↑: Increase (Reduce in Lactate Clearance) ; ↓; Decrease (Increases in Lactate Clearance); alterations in bold are statistically significant.

^{a,b}: Different letters are statistically significant between pre and post treatment.

importance of lactate has emerged in studies that were performed in association with lactic acidosis of the acute and subacute rumen (Ewaschuk et al., 2005) and in diseases, such as abomasum displacement and respiratory tract infections in cattle (Coghe et al., 2000; Figueiredo er al, 2006). Research evaluating variations in lactate levels (Kaske, 1994) and non-metabolized D-lactate levels (Lorenz and Gentile, 2014) in neonatal calves with diarrhea has appeared. It has been shown that lactate values can be used to determine the condition of strong ion acidosis (SID) in calves with diarrhea (Ewaschuk et al., 2003; Müller et al., 2012). However, no studies in the literature have evaluated lactate measurement or the effect of rehydration on lactate concentrations in calves with diarrhea. In this study, the influence of 0.9% NaCl and 1.3% NaHCO3 infusion solutions with a constant dose of 60 ml/kg on the pre-and post-treatment L-lactate and D-lactate concentrations was determined in moderately dehydrated calves with diarrhea. This is the first study comparing the effects of infusion rates of two isotonic solutions on lactate concentration and lactate clearance.

Over the last decade, it has been demonstrated that the result of a single lactate measurement can be contradictory for the interpretation of tissue perfusion (Zacher et al., 2010). Recently, research has indicated that conditions of cellular hypoxia and shock in humans must be evaluated over lactate clearance by measuring lactate concentrations at certain intervals (Vincent et al., 1983; Nguyen et al., 2004; Kamolz et al., 2005). In a study assessing the infusion rates of isotonic crystalloid and colloidal solutions of lactate clearance in patients with trauma, it was reported that the hydroxyethyl starch (HES) colloidal solution had significantly decreased lactate clearance compared with that of saline solution and resulted in less renal damage in patients with penetrating trauma; however, the relevant solutions did not cause any variation in the specified parameters in patients with blunt trauma (James et al., 2011).

In a study evaluating patients with sepsis who were admitted to an emergency clinic, different treatment groups underwent an increase in the central venous pressure (CVP) to 8 mmHg or greater. Overall, 4.5 L of isotonic crystalloid solution were administered on average for 6 h in a patient group, and the sepsis was treated. Because the study results were based on variations in sepsis levels and were not obtained by evaluating the patients who received infusions and different therapy methods, the efficacy of bolus fluid therapy on the lactate clearance could not be determined in the study (Jones et al., 2010). In another study, an average dose of 3,375 ml of crystalloid fluid altered lactate clearance at levels of 38.1% ± 34.6 and 12.0% ± 51.6 in surviving and non-surviving patients with sepsis, respectively (Nguyen et al., 2004). In our study, the ultra-rapid infusion (1 h) and the rapid infusion (3 h) of 0.9% NaCl solution at a dose of 60 ml/kg reduced the rates of lactate clearance to 36.4% and 13.6% in the calves, respectively. During ultra-rapid and rapid administration of 1.3% NaHCO3 solution to eliminate the dehydration as well as metabolic acidosis in calves with diarrhea, the variations in lactate clearance levels reduced to 31.8% in both

subgroups; however, the variations in the lactate concentrations were statistically significant in the ultra-rapid infusion group (Table 2). The relevant variations in lactate clearance were similar to those in human studies. The statistically significant reduce that occurred during ultra-rapid infusion of both solutions was considered to be due to the non-metabolizing of lactate that occurred within an hour despite eliminating dehydration and microcirculation. Furthermore, lactate clearance is closely related with capillary perfusion independent of hemodynamic variables (De Backer et al., 2006). The results in this study suggest that reducing in LC might be related to insufficient perfusion restoration, intravascular volume, and ailment energy metabolism. The outcome of statistically non-significant reduces in the clearance during ultra-rapid infusion was correlated with measurements in the third hour following fluid administration. In humans research based on the LC the sequential measurements of lactate is based on the least 6 hours intervals (Nguyen et al., 2004). In this study the time intervals between two lactate measurements were 3 hours. It is thought that the measurement of lactate clearance in such a short period of time is a limiting factor in the study.

Acute diarrhea in calves mainly causes dehydration, metabolic acidosis, electrolyte imbalance, prerenal azotemia and negative energy balance, which are independent of its etiology (Kaske, 1994; Berchtold, 1999), and these variations occur at different levels according to the duration and severity of diarrhea (Smith, 2009). Metabolic acidosis and dehydration are among the most significant complications of diarrhea (Smith, 2009). Additionally, metabolic acidosis may vary by age (Naylor, 1989). Trefz et al. (2012) found that median concentrations of D-lactate were detected at 0.8 mmol/l and 3.9 mmol/l in neonatal calves with diarrhea (age <7 days) and calves older than 7 days, respectively, and the age-dependent role of D-lactate was revealed from the development of metabolic acidosis. In this study, the level of D-lactate was in the range of 0.39-0.41 mmol/l in neonatal calves with diarrhea, and the contribution of D-lactate remained limited in the development of metabolic acidosis.

Although there are studies that have found associations between the elimination of D-lactate from the body and the clinical findings in calves (Lorenz, 2004), there are no studies regarding the association of D-lactate with renal clearance (Zello et al., 2008). In the study by Zello et al. (2008) the mean renal clearance was detected as 7.4 ± 3.4 ml/min when the blood concentration of D-lactate reached maximum levels in the calves receiving a D-lactic acid infusion. In the present study, the variations associated with infusion rates of the D-lactate concentration were observed in the blood, and the variations were measured at levels of 4.9% and 7.7% during the ultra-rapid and rapid infusions of 0.9% NaCl solution, respectively. On the other hand, variations were detected at levels of 2.6% and 4.9% during ultra-rapid and rapid infusion of 1.3% NaHCO3 solutions, respectively. In the calves treated with both infusion solutions, the low detectable concentrations of D-lactate, the low clearance of D-lactate in the blood and the low variation rates might be associated with the type of metabolic acidosis in calves with acute primary metabolic acidosis.

Several studies on different species have been conducted in the fields of human and veterinary medicine regarding the infusion rates of isotonic crystalloid solutions (Nager and Wang, 2010); however, the data that were obtained could not enhance routine practices. Because rapid infusion has risks, including hyperhydration and pulmonary edema, it has been suggested that the infusion rate should not be high. In addition to these side effects, cerebral edema, anemia and hypoproteinemia might also develop (Abeysekara et al., 2012). The maximum infusion rate of isotonic solutions has been reported to be as high as 80 ml/kg/h in dehydrated calves (Kasari, 1990; Constable, 2003). Grove-White (2007) found this rate to be risky in their study, and they argued there should be a lower infusion rate due to the risk of hyperhydration; they suggested lowering the rates to 30 or 40 ml/kg/h for a less risky and suitable infusion. In a study performed by Roussel and Kasari (1990), the best infusion rate was reported as 50 ml/kg/h in which the infusion of fluid was completed within 2 to 3 h, and the risk of hyperhydration was therefore eliminated. In present study, both 0.9 NaCl% and 1.3% NaHCO3 solutions were applied at 60 ml/kg/h for ultra-

rapid infusion and 20 ml/kg/h for rapid infusion in neonatal calves with moderate diarrhea. At the end of the infusion, the clinical findings for ultra-rapid and rapid infusions of isotonic NaCl solution in both calf groups with diarrhea were associated with an exacerbation of metabolic acidosis according to the relevant solution instead of the infusion rate.

CONCLUSION

The clearance of L-lactate and D-lactate varied with the infusion rate in calves with diarrhea, and these variations were statistically significant in both of the ultra-rapid subgroups. This study offers insight for further research on fluid therapy in calves based on the calculation of lactate clearance.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare for this manuscript.

Fig 1. Lactate concentrations in infusion groups

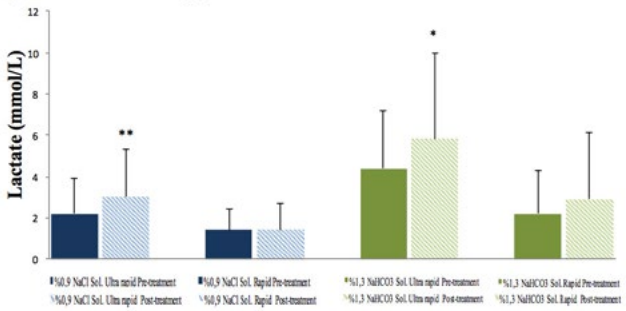
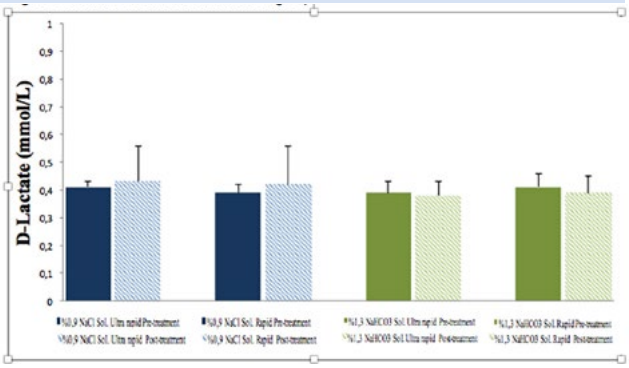


Fig 2. D-Lactate concentrations in infusion groups



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Research article
Ερευνητικό άρθρο

Study and comparison of antibacterial activities of extracts of *Zataria multiflora* and *Teucrium polium* on *Penibacillus alvei*

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ABSTRACT. In this study, the antibacterial activity of ethanol and methanol extracts of *Zataria multiflora* and *Teucrium polium* was determined against *Paenibacillus alvei* by disc diffusion method. *Paenibacillus alvei* is one secondary bacterium for the European foulbrood disease in honey bee.Minimum inhibitory concentration and minimum bactericidal concentration were determined by using the serial dilution method. For this, *Z. multiflora* and *T. polium* are collected from different areas of Iran then they are dried and extracted in lab. The antibacterial effect of alcoholic extracts of *Z. multiflora* and *T. polium* was lower than usual standard antibiotics ($P<0.01$), but the ethanol and methanol extracts of *Z. multiflora* at a concentration of 60mg/ml, have inhibitory and lethal effects on *P. alvei.* Also, 100mg/ml concentrations of ethanol extract of *T. polium* has inhibitory and lethal effects on this bacterium. But, the no one of used concentrations of its methanol extract has inhibitory and lethal effects. Results indicated that used extracts of *Z. multiflora* have the higher antibacterial effects than extract of *T. polium* on *Paenibacillus alvei*. It can be concluded that regarding the high antibacterial power of *Z. multiflora*, it is necessity to work on how they can be used in control and treatment of bacterial honey bee diseases.

Keywords: Paenibacillus alvei, Zataria multiflora, Teucrium polium, antibacterial effects, honeybee,

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INTRODUCTION

Honeybee belongs to Hymenoptera order, Apidae family and *Apis mellifera* species. The most important act that honeybees do is pollinate, in addition they produce honey, wax, polen, royal jelly, venom and propolis. Moreover, in the most flowered plants, 90% of the pollination has been done by bees (Bartomeus et al., 2014).

The European foulbrood is one of diseases which threaten the bee breeding industry especially in Iran (Shahrestani, 2015). This disease kills honeybee larvae, which could cause significant damage to the beekeeping industry. The causative agent of EFB is *Melissococcus pluton.* However, *Paenibacillus alvei* was initially thought to be the causative agent of EFB due to its isolation from affected larvae (Forsgren, 2010). The presence of P. alvei is used as an indicator of EFB as its growth produces a characteristic odour. *Paenibacillus alvei* is a gram-positive, saprophytic, aerobic, and spore-forming bacterium which causes secondary infections in already infected larvae. *P. alvei* is frequently the first indication of the presence of *M. pluton* and is almost always isolated together with the primary aetiological agent (Forsgren, 2010).

Although, the use of antibiotics is not permitted by European regulations since the year 2000, several antibiotics are still legally used against bacterial diseases of honeybees in many third countries as Iran. But due to being unaffected on the spore of mentioned bacterium, there is no final treatment. On the other hand, the remaining of pharmaceutical materials in the products of honeybees causes the dangers for the consumers of these products (Molino et al., 2011). Therefore, some effective solutions and low risk medicines should be replaced concerning the health of man and bees.

Some plants have incredible effects in treatment of infectious diseases such as antibiotics. Plants are rich in an extensive variety of secondary metabolites such as tannins, alkaloids and flavonoids which have been found to have antimicrobial properties, in vitro (Lewis and Ausubel, 2006). There are several reports about the antimicrobial activity of herbal extractions. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs and active compounds (Al Akeel et al., 2014).

Two of the plants that have been studied antibacterial properties in previous reports, are *Zataria multiflora* and *Teucrium polium*.

Zataria is a genus of flowering plant in the Lamiaceae family, first described in 1876. It contains only one known species, *Zataria multiflora*, native to south western Asia (Iran, Afghanistan, Pakistan, and Kashmir) (Manikandan et al., 2012).

Z. multiflora with the vernacular name of Avishan-e-Shirazi in Iran is used traditionally in foods as a flavor ingredient. Also, it is used as antiseptic, anesthetic, and antispasmodic, over the centuries (Hosseinzadeh et al., 2000; Ramezani et al., 2005).

The antibacterial activity of *Z. multiflora* has been shown against a number of Gram-positive and Gram-negative bacteria (Moshafi et al., 2007; Saleem et al., 2004; Abbasgholizadeh et al., 2008; Ettehad and Arab, 2007)

Teucrium polium, belong to the Lamiaceae family, is a sub-shrub and herb native to the western Mediterranean region (Albania, Spain, France, Algeria, Morocco, Tunisia). In traditional Persian medicine, *T. polium* (locally called 'kalpooreh') is used as an anti-hypertensive, anti-bacterial, carminative, anti-nociceptive, anti-inflammatory, anti-diarrhea, anti-diabetes and anti-convulsant agent. Today, the researchers investigate these properties, scientifically (Mohammadpour et al., 2015).

This paper mainly aims to determine the sensitivity value of *P. alvei* to alcoholic extracts (ethanol and methanol) of *Zataria multiflora* and *Teucrium polium*. Also, calculate the minimally inhibitory concentration (MIC) of *P. alvei* growth by the means of these two elements.

MATERIAL AND METHODS

Preparation of Zataria multiflora and Teucrium polium extracts

It has been specified that biologic activities of a sample depend on the applied methodology to prepare the desired extract. The most common used materials to provide the extract in biologic methods are ethanol and methanol.

The leaf *T. polium* and *Z. multiflora* were collected in the different region of Iran. The taxonomic identification of these plans was done by herbarium in Graduate University of Advanced Technology in

Kerman. Aerial parts of the plants dried at dark place and room temperature. Samples were ground to a fine powder and transferred into glass container and preserved until extraction procedure was performed in the laboratory. Then, 25g of these powders were separately mixed with 250ml alcohol (ethanol and methanol) and shaken by a rotator (150 rounds in 1min) at room temperature for 48 hr (Van Wyk and Wink, 2004). Afterwards, the alcoholic extract was filtered by the Whatman filter paper No.1 and the alcohol was evaporated by the use of a rotary device; finally, pure alcoholic extract was achieved. These extract of plans should be completely dried within 48 hours at 40 °C then, for providing the required concentrations; the certain weight of each dried extract was dissolved in appropriate volume of ethanol and methanol in order to obtain the desired concentrations.

Preparation of appropriate concentrations of used bacteria

Paenibacillus alvei strain NRS662, was prepared in culture medium of nutrient broth of Iranian Razi Institute. It was put in the incubator shaker at 37 °C for 48 hours in order to make the bacterial vaccine. After the growth, the bacteria were cultured in the agar nutrient medium. Afterwards, several colonies from the plates were added to Mueller-Hinton broth by the help of a sterilized loop. The inoculums of microorganisms were standardized according to the 0.5 McFarland standards which correspond to 108 colony-forming units. In next stage, 500 micro liters of resultant standard bacterial vaccine were cultured by a sterilized loop in the solid agar Mueller-Hilton culture medium.

Disk diffusion

After getting different concentrations of desired extracts (serial doubling dilutions of the extracts were prepared in a 96-well microtiter plate ranged from 10.0 mg/ml to 100.0 mg/mL), Whatman filter papers No. 2 were cut by a puncher and saturated with filter sterilized plant extracts at the prepared concentrations for 2 hours. Then, they were allowed to dry at 37°C for 5 hours. The two discs prepared in the same condition with only the corresponding volume of ethanol and methanol (1ml), were used as negative control. Each of the discs was placed on culture mediums and then the plates were incubated at 37 °C for 18 hours. The free zone around each disc indicates an area with no bacteria growth. Zones of inhibition were measured in mm.

In addition, a disc of Tetracycline (used antibiotics in the industrial beehives) was provided with a specific concentration in order to cure European foulbrood was used as positive control. All the experiment stages were repeated three times for each sample and every inhibitory zone diameter was measured by the digital caliper.

MBC (minimum bactericidal concentration) and MIC (minimum inhibitory concentration) determination

Determination of the MIC was carried out using the macro broth dilution method as recommended by the Clinical and Laboratory Standards Institute using Mueller-Hinton broth as the test medium. Overnight cultures of bacteria were diluted to yield a final concentration of 5×105 CFU/ml. The reconstituted extracts were serially diluted in two-fold in MHB medium to obtain various concentrations of the stock (10-100 mg/ml) and were assayed against the test bacteria. In the following, 1 ml of standardized inoculum (5×105CFU/ml) was added to 1ml of each extract concentration. Then, all tubes were incubated at 37°C for 18h and MIC was defined as the lowest concentration that was able to inhibit bacterial growth. Three control tubes were maintained for each test batch. These included tube containing extract and growth medium, tube containing the growth medium and inoculums, and tube containing the inoculum and standard antibiotic.

MBC values were determined using sub-culturing 150 µl of bacterial suspension from the MIC tubes into MHA plates and then incubated at 37°C for 18 h. After incubation, the concentration at which no growth was seen was recorded as the MBC.

Statistical analysis

To measure the diameter of inhibition zone, data were statistically analyzed using ANOVA method and SAS software (version 9.1) and means comparison has been done by Tukey method and proc GLM, also Pearson correlation between concentration of

Table 1. Average zones of inhibition of a standard antibiotic, Z. multiflora and T. polium ethanol and methanol extracts tested against Paenibacillus alvei cultures (mm).

Antibacteris / bacteri	Ethanol *Z. multiflora* extract	Methanol *Z. multiflora* extract	Ethanol *T. polium* extract	Methanol *T. polium* extract	Sterilized distilled water	Ethanol 96%	Methanol 96%	Standard antibiotic
Paenibacillus alvei	8.612±0.961^{b}	8.505±0.680^{b}	7.77±0.714^{bc}	6.954±0.311^{c}	*	*	*	18.07^{a}

*No growth zone. In each row, mean with the same uppercase letter is not significantly different at 5% level

the extracts and diameters of inhibition zones were calculated by proc CORR.

RESULTS

The antibacterial effect of alcoholic extracts of plants *Z. multiflora* and *T. polium* were observed against P. alvei, but it was lower than Tetracycline (P<0.01) (Table 1).

As it has been shown in Table 2, the diameter of free zone which inhibits the growth of bacteria had a direct relationship with the amount of *Z. multiflora* and *T. polium* existing in the discs. The correlations between concentration of the extracts and diameters of inhibition zones were 0.98 and 0.96 for *Z. multiflora* and *T. polium* extracts, respectively. The diameters of inhibition zones of the extracts dilutions were increased with respect to the concentration of the extracts (Pmax=0.044 and Pmin=0.001).

In order to determine the MIC and MBC values, two fold serial dilutions from concentration of 10 mg/mL to 100 mg/mL were used. Both MIC and MBC values for Z. multiflora and T. polium against P. alvei were equal to 60 and 100 mg/ml respectively.

DISCUSSION

Antibacterial effect of Z. multiflora

Today, increased resistance to antimicrobial agents has spread (Saga and Yamaguchi, 2009). So, researchers begin to evaluate the effect of plants and their active compounds for finding new and relatively low-risk compounds from different natural plants (Raskin et al., 2002). On the other hand comparing the antibacterial effect of these plants is important for choosing the most appropriate ones. In this study, the effect of alcoholic extract of two species of Labiatae family against *P. alvei* were investigated and compared.

Results indicate that the *Z. multiflora* plant extract have considerable antibacterial effects against *P. alvei* in vitro, but we do not know if the extract of this plant can control EFB in field experiments (in vivo). This research has shown that the antibacterial effect of alcoholic extract of *Z. multiflora* has been increased by the increased concentration.

The *Z. multiflora* is a plant of genus Thymus; the plants of this genus contain many phytochemical substances including terpenoids and phenolics (Fazeli et al., 2007; Guillen et al., 1998; Fatemi et al., 2012). Two of the most important secondary compounds of this genera are fairly known; volatile oils and phenolic compounds. On the other hand the most pharmacological effects of Thymus genus are because of these two classes of compounds (Simeon de Bouchberg et al., 1976; Stahl-Biskup and Saez, 2002). Among terpenoids, the phenolic terpenes; thymol and carvacrol, rank highest in importance (Shafizade, 2002). Studies have shown that these phenolic compounds, especially carvacrol (a main constituent of Avishan-e-Shirazi essential oils) have a high antibacterial effect (Nejad Ebrahimi et al., 2008; Baydar et al., 2004). Moreover, it is possible that low-concentration components or interaction between some of the constituents are responsible for the antibacterial effects (Stanković Nemanja et al., 2011).

Antibacterial effect of T. polium

T. polium is one of the most important medicinal plants that extensively used in traditional medicine in Iran. The previous researches have shown that ethanolic and methanolic extracts of *T. polium* aerial parts are effective against both gram positive and gram negative bacteria. However, studies have indicated that hydroalcoholic extract of *T. polium* is more effective in positive gram bacteria

Table 2. Average zones of inhibition in different Concentrations of *Z. multiflora* and *T. polium* extracts against *Paenibacillus alvei* cultures

Antibacterial materials Concentration (mg/mL)	Ethanol *Z. multiflora* extract (mm)	Metanol *Z. multiflora* extract (mm)	Ethanol *T. polium* extract (mm)	Methanol *T. polium* extract (mm)
10	6.8^{iA}	6.5^{hB}	6^{hC}	6.5^{fB}
20	7.2^{hA}	6.75^{ghB}	6^{hD}	6.5^{fC}
30	7.5^{gA}	7.06^{gB}	6^{hD}	6.5^{fC}
40	8^{fA}	7.57^{fA}	6.57^{gB}	6.78^{eB}
50	8.2^{fA}	8.09^{eA}	7.73^{fB}	6.85^{deC}
60	8.5^{eA}	8.7^{dA}	8.13^{eB}	6.97^{dC}
70	9^{dB}	9.16^{cA}	8.76^{dC}	7^{dD}
80	9.32^{cB}	9.8^{bA}	9^{cC}	7.18^{cD}
90	10.28^{bA}	10.08^{bA}	9.2^{bB}	7.52^{bC}
100	11.32^{aA}	11.34^{aA}	10.31^{aB}	7.74^{aC}

Note: In each column, mean with the same lowercase letter is not significantly different at 5% level. In each row, mean with the same uppercase letter is not significantly different at 5% level

in comparison with gram-negative ones due to the difference of cellular wall structure or other genetic factors of gram-positive and gram-negative bacteria (Darabpour et al., 2010).

The antibacterial activity of the *T. polium* extract might be due to its richer in the phenols and flavonoids Compounds (Chedia et al., 2013). It is known that polyphenols are bioactive molecules. These biological activities are related to the molecules structures; by their hydroxyl groups or by phenolic ring, phenolics compounds have capacity to link with proteins and bacterial membrane to form complexes (Zongo et al., 2011). The antibacterial compounds of plants may inhibit bacterial growth by different mechanisms. Therefore, these plants may have a significant clinical value in treatment of resistant bacterial strains (Sarac and Ugur, 2007).

Antibacterial properties of ethanolic and methanolic extracts of *T. polium* against some of clinical pathogens were studied by Darabpour et al. (2010), the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Salmonella typhi* was 40mg/mL and *Bordetella bronchiseptica* and *Bacillus anthracis* was 10 mg/mL. The minimum bactericidal concentration (MBC) against *Bacillus anthracis* was 10 mg/mL while against other species were not found (>200 mg/mL).

Mahboubi et al. (2014) have investigated the antibacterial effect of *Zataria multiflora* against four foodborne and four other bacteria including *Staphylococcus aureus, Shigella dysenteriae, Salmonella typhimurium, Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis* and *Pseudomona aeruginosa*. This study showed that Z. multiflora was effective with MIC values between 0.78-3.125 mg/mL against all of the bactria.

Haider et al. (2014) have studied antibacterial activity of northern Ontario medicinal plant extracts. In their study, the antibacterial activity (in vitro) of the leaf or flower extracts of Anaphalis margaritacea *L., Grindelia squarrosa* (Pursh), *Apocynum androsaemifolium L., Arctostaphylos uva-ursi L., Cornus canadensis L.* and *Xanthium strumarium L.* (medicinal plants) was analyzed through the hole-plate diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays against *Escherichia coli, Aeromonas caviae, Paenibacillus alvei, Micrococcus luteus, Mycobacterium avium* subsp. avium and *Bacillus cereus* bacteria. The leaf and flower extracts of *Anap.* margaritacea and *G. squarrosa* have a significant antibacterial activity against all the bacteria tested, with inhibition of *A. caviae*, *P. alvei* and *M. luteus* within 1-12 h of incubation at MBC.

In present research, the antibacterial effects of ethanol and methanol extracts of *Z. multiflora, T. polium* and standard antibiotic concerning *P. alvei* in honeybees were compared. Results have shown that there is a meaningful difference between the effects of used extracts and common antibiotic on P. alvei ($P<0.01$). As well, the ethanol and methanol extracts of *Z. multiflora* were significantly higher than ethanol and methanol extracts of *T. polium* ($P<0.01$). However, four extracts have significant antibacterial activities on the used bacteria.

In previous studied, it has been demonstrated that regarding European foulbrood, tetracycline antibiotics had stronger effects on European and American foulbrood diseases (Mutinelli, 2003). The water extract of 10 plant species were tested by González and Marioli (2010) as inhibitors for the growth of *Paenibacillus larvae*, the causative agent of American Foulbrood. *Achyrocline satureioides, Chenopodium ambrosioide, Eucalyptus cinerea, Gnaphalium gaudichaudianum, Lippia turbinata, Marrubium vulgare, Minthostachys verticillata, Origanum vulgare, Tagetes minuta* and *Thymus vulgaris* were included in their study. Results showed the growth of almost all the *P. larvae* strains tested was inhibited by these extracts. However, no research has been carried to study antibacterial effect of plant extracts against EFB or AFB in field experiments.

CONCLUSION

Finally, this research results indicated that ethanol and methanol extracts of *Z. multiflora, T. polium* have the antibacterial effects on *Paenibacillus alvei* under laboratory conditions, and if more studies are conducted, the main active substance can be achieved by the means of these products. By the way, antibacterial effect of plant extracts may be different under field experiments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Laparoscopic castration in stallions with two different testicular blood vessels occluding techniques

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ABSTRACT. Castration of horses is indicated for many reasons and could be performed with variable techniques. Laparoscopic castration is an improved choice. Laparoscopic castrated horses have better cosmetic appearance, return rapidly to normal physical status and have little postoperative complications. The main challenges during laparoscopic castration are obtaining an optimal visualization of the internal inguinal orifices and their content of vas deferens and testicular blood vessels and appropriate occlusion and transection of the testicular blood vessels to obtain avascular atrophy of the testicles that remains in situ within scrotum. Laparoscopic castration was performed in horses lied in lateral recumbency for better visualization of the target structures and two methods for vascular occlusion were compared. Endo-clipping is effective testicular vessels occluding method and more reliable than bipolar cauterization. Lateral recumbency layout decreased risks of hemodynamic changes during surgery and provided optimal visualization of the surgical field.
Keywords: Laparoscopy, Castration, Horses, Clipping.

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INTRODUCTION

Complications accompanying equine castration are the most common cause for searching more reliable techniques. Unlike traditional castration techniques, which possess tissue damage and followed by variable complications, laparoscopic surgery is performed through small incisions had shown minimal tissue invasiveness, short hospitalization and minimal post-operative complications (Searle et al., 1999). Laparoscopic castration techniques have been reported in horses (Fischer, 1991; Fischer & Vachon, 1992; Wilson et al., 1996; Fischer & Vachon, 1998; Ragle, 1998; Walmsley, 1999; Walmsley, 2003; El-Khamary et al., 2017). Improvements dedicated to laparoscopic techniques involves finding better performing position and accurate testicular vessels occluding techniques. Laparoscopic castration performed in standing position (Davis, 1997; Hendrickson & Wilson, 1997), dorsal recumbency (Wilson et al., 1996). Placing the horses in lateral recumbency during laparoscopic castration surgery had shown high efficiency (El-Khamary et al., 2017). The key point of castration is occlusion of the testicular blood vessels and prohibition of the blood supply to the testicles followed by removing or keeping the testicles in situ are both reported (Fischer & Vachon, 1998; El-Khamary et al., 2017). Electrocoagulation monopolar and bipolar techniques, pre-tied ligating loops and clipping are the most common laparoscopic vessel occluding techniques (Alsafy et al., 2013). The aim of the present study was to a) describe laparoscopic castration technique in lateral recumbency as a modified layout and to b) compare two laparoscopic vessel occluding approaches; bipolar cauterization and new laparoscopic clipping method.

MATERIALS AND METHODS

The present study was performed on nine premature horses (6-8 months), all horses were healthy and submitted for castration in order to avoid unwanted masculine behavior. Four horses were referred from one single farm the other five horses were sporadic. Surgeries were performed at the era between 2014 and 2017.

Food was withheld 36 hours prior surgery and the flanks and ventral abdomen were clipped and prepared for aseptic surgery. All horses received sedative (Xylaxine; Xylaject, ADWIA Pharma, Egypt, 0.5 mg/kg) administered intravenously, then, generally anesthetized (ketamine; Ketamine 5%, Sigmatech Pharma, Egypt, 2.2 mg/kg) administered intravenously. Anesthesia was maintained with repeated half dose bolus when required via intravenous rout according to (Staffieri & Driessen, 2007).

Member horses were lied in lateral recumbent position and secured to the surgery table with the uppermost hind limb abducted and elevated outward. Infiltration of 10 ml of local anesthetizing solution (Lidocaine; Depocaine 2%, DEPIKY PHARMA, Egypt) into two points; umbilicus and ventral inguinal region, the main and secondary portal points according to (El-Khamary et al., 2017). Standard laparo-insufflation was created to maintain sufficient intraabdominal space for surgery. Main port 10-mm cannula was introduced via an incision through the umbilicus and used to incorporate the laparoscope. Another 10-mm secondary portal cannula is inserted into the abdominal cavity at the ventral inguinal point. The uppermost abdominal and pelvic cavities were explored and the ipsilateral testicular vessels were located.

Laparoscopic castration by bipolar cauterization of TBV:

Laparoscopic castration with bipolar cauterization of the TBV was applied to four horses. Bipolar forceps used to coagulate and cut the TBV by applying frequent jets of high frequency (150 watts) bipolar current. Continuous bipolar dissection of the TBV from peritoneum and complete severing of the vas deferens was performed (figure 1).

Laparoscopic castration by clipping of TBV:

Laparoscopic castration by clipping of the TBV and vas deference was performed in five horses according to (El-Sherif, 2013). Four medium-large titanium clips mounted on 10-mm endo-clip applicator applied over the course of the testicular blood vessels, then, transected in between with scissors. Further application of two clips over the course of the vas deferens and transection with scissors (Figure 2).

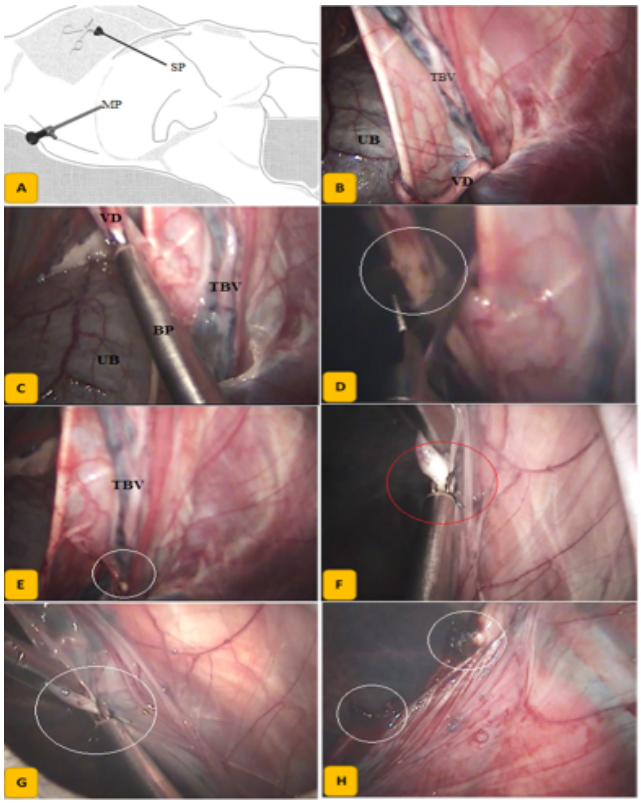

Figure 1. Laparoscopic castration by bipolar cauterization of TBV. Horse layout in lateral recumbency; main port for laparoscope in umbilicus (MP), secondary port for instrument (SP) (A), inspection of the testicular blood vessels (TBV) and vas deferens (VD) (B), bipolar cauterization of VD by bipolar electrode (BP) (C), severing of the VD (white circle) (D), severed stump (white circle) (E), bipolar cautery of TBV (red and white circles) (F&G), after complete severing of TBV, stumps in white circles (H).

Intra and Post-operative evaluation:

Instant exploration for bleeding was performed in all animals. Feasibility and surgical rating was assessed to scale inspired from that build by Martini et al., 2014 (table, 1), operative time, tools required, degree of visibility and easiness were documented for each procedure, final judgement was marked down. Reliability of the laparoscopic technique was evaluated according to scale grading the surgeon skills that mimic scale reported by Vassiliou et al., (2006) (table, 2). Three months follow up for all horses including; disappearance of masculine behavior, presence of genital or testicular disorders, general health status and periodic ultrasonographical evaluation of the testis, inguinal and ventral abdomen regions. Animal value/ cost effectiveness was also evaluated.

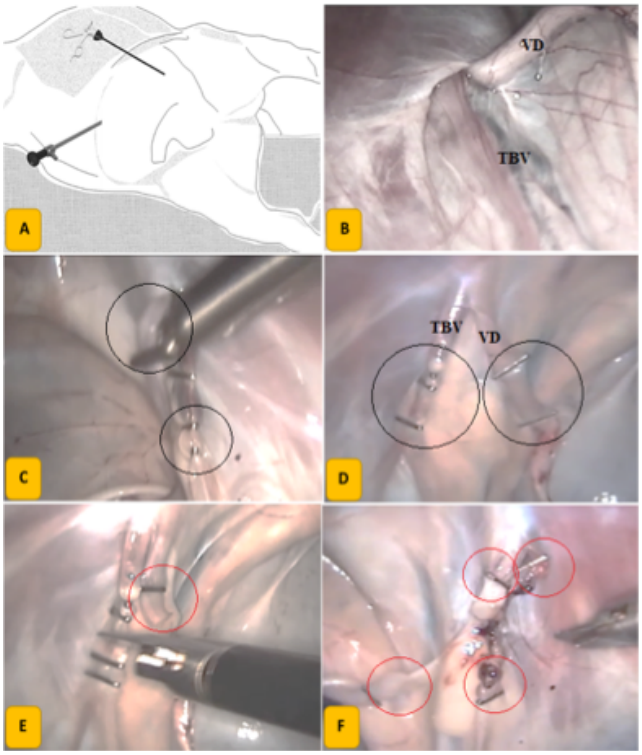

Figure 2. Laparoscopic castration with clipping and severing of TBV and VD. Horse layout in lateral recumbency (A), anatomical identification of TBV and VD (B), direct application of medium/ large titanium clips on the course of TBV and VD by endo-clip applicator (C), complete clipping (D), severing of TBV and VD by endo-scissors (E), complete transection of TBV and VD (F).

Table 1: Surgical feasibility rating scale.

Parameter	score
Disability of surgery due to patient related causes (movements, pain manifestation or orientation)	Poor/ good/ optimal
Ability to obtain visible surgical field	
Enough working field for performing surgery	

Table 2: Surgical reliability rating scale.

Parameter	score
Ability to handle structures	Poor/ good/ excellent
Ability to apply monopolar cauterization and ascertain of process	
Ability to apply titanium clips and ascertain process	

RESULTS

Xylazine-Ketamine general anesthesia was adequate for all nine surgeries. The mean surgical time was 39±4 minutes for laparoscopic castration with bipolar cauterization and 14±2 minutes for laparoscopic castration with clipping of the TBV. Laying animals in lateral recumbency was easy and comfortable for the operator. Trendelenburg position was not required, lateral recumbency with up to -10 mm/hg- pneumoperitoneum provided suitable operating space for performing surgery. laparoscope at the umbilicus was optimal to visualize the entire pelvic cavity and internal inguinal region.

Laparoscopic castration with bipolar cauterization of TBV:

Trials to grasp and cauterize the TBV or VD through a single tool failed, another secondary port was inserted through the abdomen five cm distal to the inguinal secondary portal. The uppermost portal used to introduce endoscopic Allis grasper and the lower used to introduce the bipolar electrode. Several attempts were done to cauterize the VD, effective coagulation was determined visually by complete dryness and changing the color into fade white. Bipolar current was optimized for coagulation to avoid cutting of improperly occluded vessels. Otherwise, cutting was performed using endo-scissors alternatively after coagulation. Intraoperative complications were improper fogy vision of the operative field due to smokes generated as a result of tissue burn within sealed abdomen. Operators needed about 4 minutes to retain visualization for the next attempt of cautery or cutting. Tissue adhesion to the metallic shaft of the bipolar electrode was recorded in two horses and caused extra peritoneal damage. Bleeding from the TBV was recorded in 3 cases due to improper coagulation. After further attempts, bleeding was controlled. The VD was coagulated and severed in fewer cautery turns and easier than the TBV. Intra-operative complications are grouped in figure 3.

Although further administration of anesthetic booster doses in all horses of the group, there were no disabilities related to the animals during the surgery. Visualization of the surgical field was optimal before starting bipolar cauterization and changed to poor after each cautery attempt to good after about 3-4 minutes later. Ability to handle structures with one tool was poor.

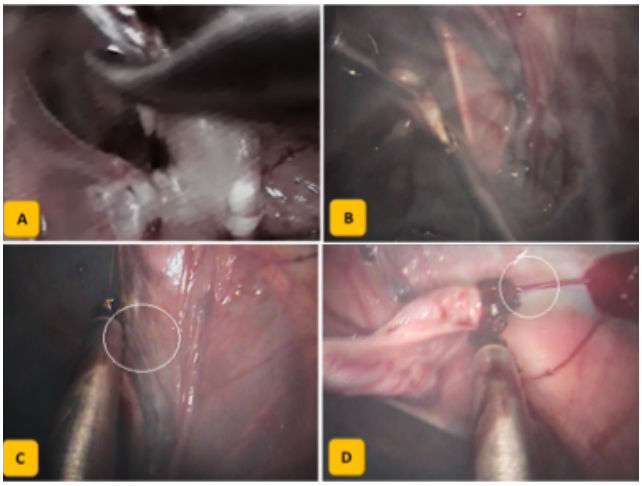

Figure 3. Intra-operative complications associated with bipolar cautery of TBV and VD. Some are technical; haziness of visual field during current incorporation (A), fogy vision of the operative field due to smokes generated from tissue burn (B), and some are related to faulty use; adhesion between the electrode and adjacent tissue (C), bleeding due to improper coagulation of large diameter TBV (D).

Laparoscopic castration with clipping of the TBV:

Application of medium/ large endo-titanium clips to the course of both the VD and TBV was easy and reliable. There was no need to use other secondary portal for supporting or grasping tool. Severing of the VD and TBV was performed using endo-scissors after complete clipping of both structures. The procedure was reliable, feasible, accurate and time saving. There were no intra-operative complications.

The overall reliability and feasibility evaluation of laparoscopic castration with bipolar cauterization or clipping are summarized in table 3.

DISCUSSION

Although diagnostic laparoscopic surgery in veterinary field in Egypt had been introduced in mid nineteens, clinical interventional applications of laparoscopy were not implicated until the second millennium (El-Sherif, 2008; El-Khamary, 2011; El-Sherif, 2013; El-Sayad, 2016; El-Khamary et al., 2017). Due to the high importance of that field, the present

Table 3: Reliability and feasibility of laparoscopic castration.

Parameter	Laparoscopic castration with bipolar cautery	Laparoscopic castration with clipping
Disability of surgery due to patient related causes	*Optimal*	*Optimal*
Ability to obtain visible surgical field	*Optimal -poor- good*	*Optimal*
Enough working field for performing surgery	*Good*	*Optimal*
Ability to handle structures		
Ability to apply monopolar cauterization and ascertain of process	*Good - poor*	----
Ability to apply titanium clips and ascertain process	----	*Optimal*

study similar to all previous trials is related to gynecological laparoscopic applications. To our knowledge, this is the first study reports clinical nonexperimental application of interventional laparoscopy in horses in Egypt.

Laparoscopic surgery is performed under the effect of sedation and local infiltration anesthesia in standing horses (Fischer, 1991) and under general inhalant anesthesia in dorsal recumbency layout (Fischer & Vachon, 1992). Successful attempts to perform under the effect of total intravenous anesthesia (TIA) were reported (El-Sherif, 2013). In order to reserve the hemodynamic balance and decrease the pressure on thorax during TIA, animal layout was changed to lateral recumbency (El-Sherif, 2013; El-Khamary et al., 2017). Gathering fast performing by skilled well-trained surgeon and putting the animal in lateral recumbency resulted in feasible, safe and reliable laparoscopy. Another one advantage is proper visibility of the pelvic contents especially the ipsilateral uppermost region where the inguinal canal lies. Fasting of the animals for 36-72 hours is a must to avoid entrapment of large intestines within the surgical field (El-Khamary et al., 2017). In adequate intestinal emptying results in occupation of the surgical field and need to put the horse into anterior Trendelenburg position with the head down. Thirty-six hours fasting was adequate to empty intestines in young horses aging up to 8 months. The ability to visualize both inguinal areas was possible in all horses, otherwise, shaking the animal and tilting the abdomen into cranial Trendelenburg with 15o was required almost in all horses before starting surgery to provide better visualization of the surgical field.

Bipolar electrode is provided with 5mm outer diameter shaft, so, the 10-mm secondary port should be provided with an extra adaptor. It was hard to cauterize the target blood vessels without grasping them away from the neighboring tissue. First attempt to use bipolar electrode alone without assistant tool resulted in adhesion between the outer metallic shaft of the tool and peritoneum (figure, 3-C). insertion of extra 5-mm secondary portal made cauterization easier. Alike results reported by (El-Khamary, 2011 and Alsafy et al., 2013) the bipolar current could not achieve reliable coagulation of testicular blood vessels. Otherwise good coagulation of vas deference and its accompanying blood vessel was obtained after two or three shots. Further frequent shots were needed after severing the TBV to control bleeding. Endoscopic clipping is a reliable occluding method of large tubulated structures. It is used to occlude cystic duct and cystic blood vessels during human laparoscopic cholecystectomy (Troidl et al., 1992). Endoscopic clipping was used effectively to occlude the TBV to induce in-situ castration in dogs (El-Sherif, 2008) and in equines (El-Khamary et al., 2017). Applying four titanium clips to the course of the TBV and two on the course of the VD was feasible through a single port at the inguinal region, easy, reliable and effective. There was no bleeding after severing the TBV or VD. Another theoretical advantage of endo-clipping is complete prohibition of blood streaming to the testicles either of suggested revascularization of the TBV.

CONCLUSIONS

Laparoscopic castration is an effective alternate to conventional methods that is reliable and more cosmetically acceptable. Endo-clipping is a reliable and accurate large vessel occluding method that may be used safely in laparoscopic castration procedures. Laying horses in lateral recumbency during laparoscopic castration provide the operator with a wide visible field and permits good manipulation of target structures with minimal effects on the hemodynamic balance. Laparoscopic experimental studies should be applied and evaluated in clinical studies.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare for this manuscript.

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Case report
Κλινικό περιστατικό

Bilateral primary eyelid meibomian gland epithelioma in a dog: a case report.

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ABSTRACT. In this paper an extremely rare case of primary bilateral eyelid meibomian gland epithelioma in a dog is presented. Meibomian tumors arise from the meibomian (tarsal) glands on the inner aspect of the eyelid, and meibomian gland adenomas and adenocarcinomas are the most frequent neoplasia arising from those glands. A 10-year-old male dog was admitted, with ophthalmic signs of periorbital swelling of the right eye. During the last two years, the dog had a progressive engorgement of the upper eyelid of the right eye, and during the past few months, strabismus and exophthalmos were also noticed, as well as a slighter enlargement on the lower eyelid of the left eye. Upon ophthalmic examination strabismus and periorbital swelling at the lateral canthus of the right eye, accompanied by mild exophthalmos and lagophthalmos, were recorded. Also, deep ulcerative keratitis with corneal oedema and neovascularization were noticed. Intense chemosis, epiphora and a small pinkish mass arising from the palpebral conjunctiva of the left lower eyelid were also seen. Neoplasia of both eyelids was suspected, and a series of diagnostic examinations were carried out. Orbital exenteration of the right eye was performed in the first place, followed by surgical removal of the mass of the left eyelid one month later. Based on clinical and histopathological findings, meibomian gland epithelioma was diagnosed. No relapse or metastases (local or distant) were observed in re-examinations within 18 months after the surgery. This study shows that although meibomian gland epithelioma is rare, it should be considered in the differential diagnosis of cases of orbital and eyelid tumors. Even though this tumor shows low malignancy, prompt surgical intervention and extended excision is the treatment of choice, in order to preserve vision in some cases. To the authors'

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knowledge, the present case is the first report of bilateral meibomian gland epithelioma in the veterinary literature.
Keywods: Bilateral eyelid tumor, meibomian epithelioma, tarsal tumor, eyelid, dog.

ΠΕΡΙΛΗΨΗ. Σε αυτή την εργασία γίνεται αναφορά σε ένα σπάνιο κλινικό περιστατικό με αμφοτερόπλευρο πρωτογενές επιθηλίωμα των μεϊβομιανών αδένων σε σκύλο. Οι νεοπλασίες των μεϊβομιανών (ταρσαίων) αδένων προέρχονται από τη βάση των αδένων αυτών, στον ταρσό του βλεφάρου. Τα αδενώματα και τα αδενοκαρκινώματα αποτελούν τις συχνότερες νεοπλασίες των ταρσαίων αδένων. Σκύλος, αρσενικός, ηλικίας 10 ετών, προσκομίστηκε με μία περιοφθαλμική διόγκωση στο άνω βλέφαρο του δεξιού οφθαλμού που τα τελευταία δύο χρόνια αναπτυσσόταν σταδιακά, ενώ στον αριστερό οφθαλμό παρατηρήθηκε από τον ιδιοκτήτη μία μικρότερη διόγκωση στο κάτω βλέφαρο του αριστερού οφθαλμού. Κατά την οφθαλμολογική εξέταση διαπιστώθηκε στραβισμός, περιοφθαλμική διόγκωση στον έξω κανθό του δεξιού οφθαλμού, που συνοδευόταν από ήπιο εξόφθαλμο και λαγόφθαλμο. Παράλληλα υπήρχε εν τω βάθει ελκώδης κερατίτιδα, με οίδημα και νεοαγγείωση στον κερατοειδή. Στον αριστερό οφθαλμό διαπιστώθηκε έντονη χύμωση, επιφορά και η παρουσία μίας μάζας που προερχόταν από τον επιπεφυκότα του κάτω βλεφάρου. Με βάση την κλινική εικόνα τέθηκε υποψία νεοπλάσματος και στους δύο οφθαλμούς και διενεργήθηκαν διάφορες διαγνωστικές εξετάσεις. Στη συνέχεια έγινε εξόρυξη του δεξιού οφθαλμού και ένα μήνα μετά ακολούθησε χειρουργική αφαίρεση της μάζας του αριστερού οφθαλμού. Με βάση την κλινική και την ιστοπαθολογική εικόνα του περιστατικού, τέθηκε η διάγνωση του επιθηλιώματος των μεϊβομιανών αδένων. Σε επανεξετάσεις που έγιναν μέχρι και 18 μήνες μετά δεν παρατηρήθηκε υποτροπή, ούτε διαπιστώθηκαν επιχώριες ή απομακρυσμένες μεταστατικές εστίες. Το επιθηλίωμα των μεϊβομιανών αδένων, αν και σπάνιο, θα πρέπει να συμπεριλαμβάνεται στη διαφορική διάγνωση των όγκων του οφθαλμικού κόγχου και των βλεφάρων. Αν και οι μεταστάσεις είναι σπάνιες, η έγκαιρη χειρουργική εξαίρεση αποτελεί την θεραπεία εκλογής, προκειμένου να διατηρηθεί ο βολβός και η όραση σε κάποιες περιπτώσεις. Στην παρούσα εργασία γίνεται αναφορά για πρώτη φορά σε αμφοτερόπλευρο επιθηλίωμα των μεϊβομιανών αδένων.

Λέξεις ευρετηρίασης: Αμφοτερόπλευρη νεοπλασία βλεφάρων, επιθηλίωμα μεϊβομιανών αδένων, νεοπλασία ταρσαίων αδένων, βλέφαρο, σκύλος.

INTRODUCTION

Eyelid tumors, although infrequent in the domestic species, are of major consideration when it comes to periorbital proliferative swellings. The most frequent canine eyelid neoplasms involve meibomian gland adenoma, squamous papiloma, meibomian adenocarcinama, melanoma (benign and malignant), histiocytoma, mastocytoma, basal cell carcinoma, squamous cell carcinoma, fibroma, fibropapilloma, lipoma and others (Krehbiel and Langham, 1975; Roberts et al., 1986). Studies have shown that epithelial tumors seem to be more prevalent than the tumors of mesenchymal origin (Krehbiel and Langham, 1975). Most of the eyelid tumors in dogs are locally minimally invasive (Frans and van der Woerdt, 2013). In addition, several retrospective studies suggest that the majority of the eyelid tumors (73.3%-87.8%) proved to be benign (Krehbiel and Langham, 1975; Roberts et al., 1986), however, malignant tumors often recurred locally but did not have a high frequency of metastasis (Krehbiel and Langham, 1975).

Meibomian tumors arise from the meibomian (tarsal) glands on the inner aspect of the eyelid (Maggs, 2007), and meibomian gland adenomas and adenocarcinomas are the most frequent neoplasia arising from those glands (Krehbiel and Langham, 1975; Roberts et al., 1986). Benign meibomian gland tumors present as focal or multifocal nodular masses. A meibomian gland adenoma is made up of fully differentiated meibomian glandular tissue, including the holocrine secretory cells and the keratinizing ducts. On the other hand, meibomian gland epitheliomas consist of poorly differentiated basal cells and rarely show sebaceous or squamous differentiation. Similarly, epitheliomas are more likely to be pigmented and they are slightly larger and probably located deeper in the lid margin dermis than adenomas. Eyelid tumors in general, may cause problems related to their contact with

the corneal surface causing irritation, or may be considered a cosmetic problem (Dubielzig et al., 2010). Advanced meibomian adenomas or adenocarcinomas may ulcerate or hemorrhage. Also, local irritation resulting in blepharospasm, epiphora, conjunctival hyperemia, corneal vascularization, and pigmentation are reported. Therefore, early surgical removal of eyelid tumors is the treatment of choice (Frans and van der Woerdt, 2013).

The dog described in this report is an extremely rare case with bilateral primary meibomian gland epithelioma. To the authors' knowledge, there is no similar case reported in the veterinary literature.

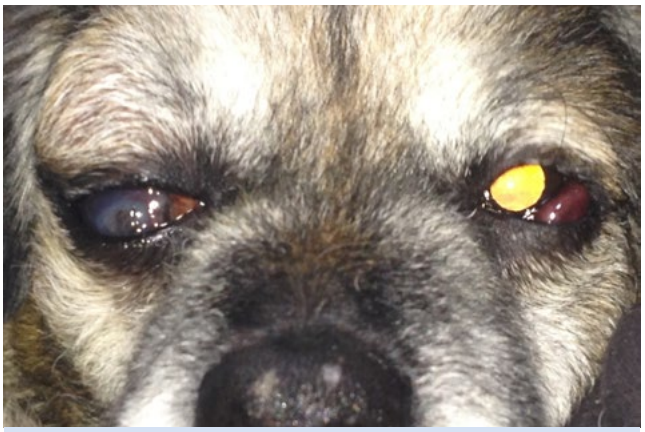

Fig 1: A dog with periorbital swelling and strabismus of the right eye, and oedema of the lower eyelid of the left eye.

CASE HISTORY

A 10-year old spayed, crossbreed, male dog was presented to the Comparative Ophthalmology Unit of the Companion Animal Clinic, School of Veterinary Medicine, Aristotle University of Thessaloniki, with ophthalmic signs of exophthalmos of the right eye and mild swelling of the lower eyelid of the left eye. According to the owner, during the last two years, the dog had a progressive engorgement of the upper eyelid of the right eye, whereas during the past few months strabismus and exophthalmos were also noticed. A smaller but yet significant enlargement on the lower eyelid of the left eye, was also noticed. The dog had been treated during the past two years several times by private practitioners with topical antibiotics such as tobramycin and fucidic acid, as well as with systemic antibiotics: amoxycillin and clavulanic acid, along with corticosteroids, without any significant improvement.

Upon thorough ophthalmic examination, strabismus and periorbital swelling in the lateral canthus of the right eye, accompanied by mild exophthalmos and lagophthalmos were recorded. Also, mucopurulent discharge, conjunctival and scleral congestion, epiphora, deep ulcerative keratitis along with corneal oedema and neovascularization were noticed. Furthermore, intense chemosis, epiphora and a small pinkish mass arising from the palpebral conjunctiva of the lower eyelid of the left eye was seen. The globe and rest of the ophthalmic structures appeared normal (Fig. 1). On general physical examination nothing abnormal was noticed.

Neoplasia of both eyelids was strongly suspected and, for definite diagnosis, a series of diagnostic tests were performed. More specifically, complete blood count and biochemistry profile along with urinalysis were carried out, but were not diagnostic. Aside a mild neutrophilia (9.3K/μl, Reference Values: 3.9-8.0K/μl) and a slightly elevated mean platelet volume (MVP) 11.7fl (Reference Values 5.4-9.2), the rest of the values were within normal range. An ocular ultrasonography and computer tomography were performed, confirming the presence of a mass in the upper peribulbar space of the right eye. Lastly, abdominal and thoracic radiographs were taken to rule out metastasis in the respective cavities and proved to be none. Fine needle aspiration of the mass for cytologic examination was performed under general anesthesia prior to surgery, but it was not diagnostic as no pathological cells were identified.

Orbital exenteration was the treatment of choice for the right eye, since the damage to the eye was far too progressed. The dog was anesthetized using dexmedetomidine (0.009mg/kg b.w., intramuscularly, Dexdomitor, Zoetis) for premedication, propofol (2mg/kg b.w., intravenously, Vetofol, Norbrook) for the induction and isoflurane (Iso-Vet, Piramal Healthcare) for maintenance of anesthesia. A nonsteroidal anti-inflammatory drug (NSAID), meloxicame (Metacam, Boehringer Ingelheim) was administered pre-surgically at a dose of 0.1mg/kg b.w., intravenously. The transpalpebral approach was the surgical procedure of choice, with excision of the globe and the periorbital tissues, with extended removal of the mass originating from the upper eyelid tarsal area (Fig. 2). During the surgery fine needle aspiration samples were obtained from the mass, in order to have an early diagnosis. One month later, surgical removal of the mass located in the palpe-

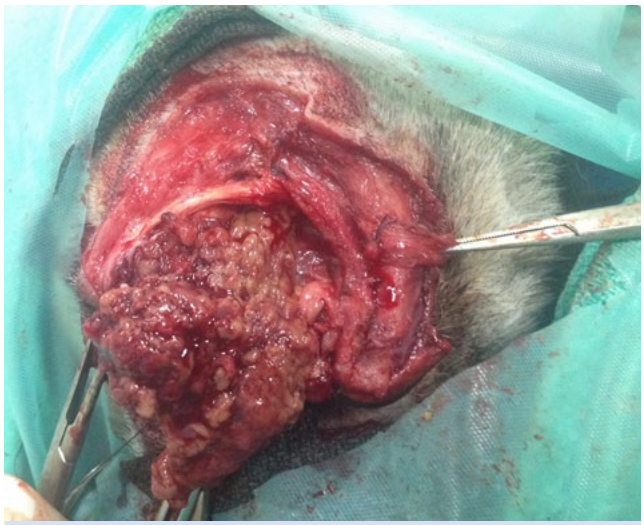

Fig 2: Excision of the globe and periorbital tissues, with extended removal of the tumor of the right eye.

bral conjunctiva of the left lower eyelid was scheduled. The decision to perform the second surgery one month later was based on the age of the dog, the duration and severity of the first surgery, and the time required for the histopathological results of the mass. The dog was anesthetized using acepromazine (0.05mg/kg b.w., intramuscularly, Acepromazine Maleate, VEDCO) and butorfanol (0.1mg/kg b.w., intramuscularly, Butomidor, Richerpharma) for premedication, propofol for the induction and isoflurane for maintenance of anesthesia and meloxicame was given as well (at the doses mentioned before). After incision on the palpebral

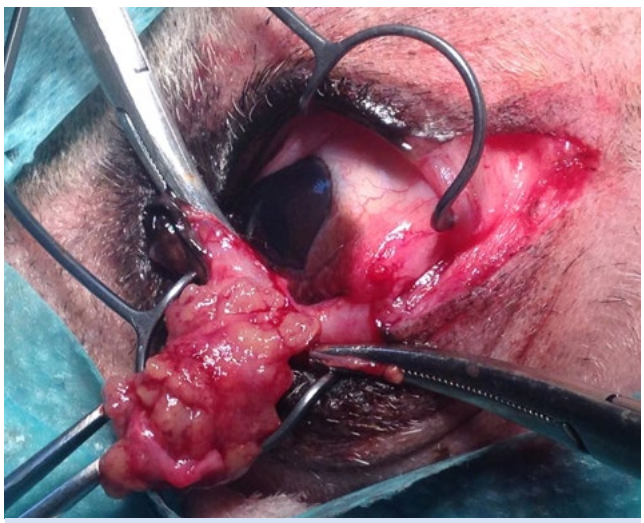

Fig 3: Surgical removal of the mass of the left eye. After incision on the palpebral conjunctiva of the lower eyelid of the left eye, the mass was easily separated from the surrounding tissues and completely excised.

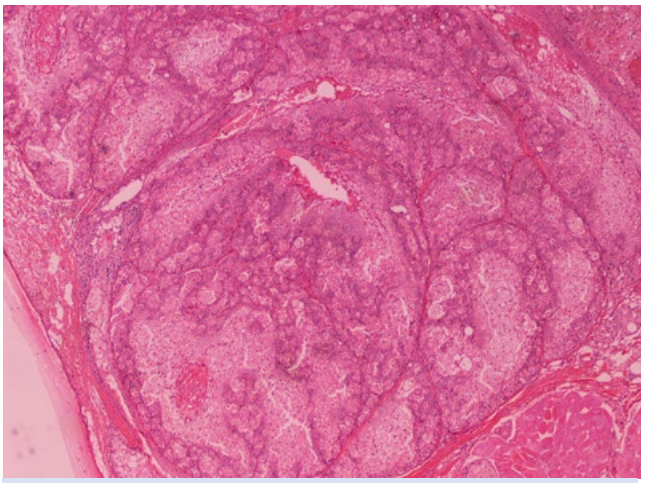

Fig 4: Lobular pattern of the mass, and presence of sheets of undifferentiated basal reserve cells with rare sebaceous differentiation. HE, original magnification ×4.

conjunctiva of the lower eyelid, the mass was easily separated from the surrounding tissues and completely excised (Fig. 3). The right eye globe along with several tissue samples from the mass and periorbital tissues, as well as the mass from the left eye were preserved in 10% formaldehyde and submitted for histopathological examination. Post operatively, after both surgeries, broad spectrum antibiotics; amoxycillin and clavulanic acid (12.5mg/kg b.w., BID for 8 days, per os, Sy-

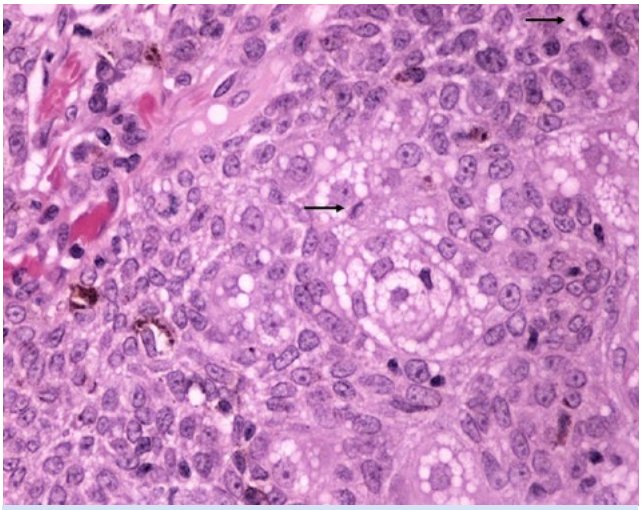

Fig 5: Growth of basaloid cells with scattered aggregates of sebocytes, and melanocytes. Some mitotic figures are seen (arrows) in reserve cells. HE, original magnification ×200.

nulox, Merial) and clindamycin (11mg/kg b.w., SID for 8 days, per os, Antirobe, Sanico n.v.), as well as NSAID meloxicame (0.1mg/kg b.w., SID for 3 days,

Metacam, Boehringer Ingelheim) were administered, and an Elizabethan collar was placed.

Fine needle aspiration samples that were taken during the surgical procedure proved to be inconclusive caused by suboptimal preparation and bloody smears. The histopathological appearance of the examined samples revealed the presence of sheets of undifferentiated basal cells with rare sebaceous differentiation. Focally, scattered melanocytes were detected. Based on gross and microscopic findings, the present case was diagnosed as bilateral meibomian gland epithelioma (Fig. 4 and 5).

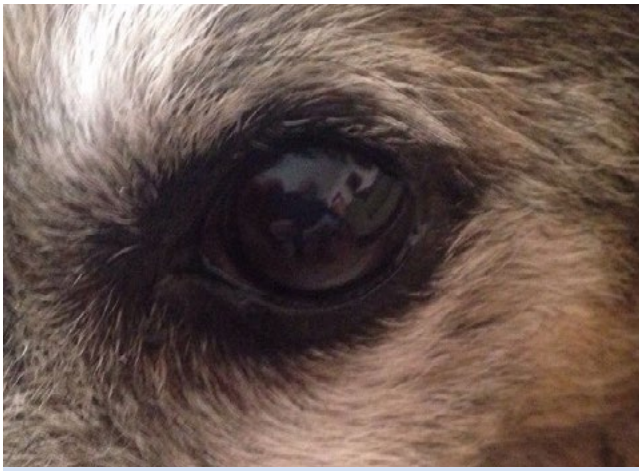

Fig 6: Left eye, 3 months after surgical removal of the mass. The lower eyelid appears normal, without any anatomical deformities.

Re-examinations were scheduled a week, one and three months after each surgery and thereafter every six months. The surgical site of the right eye had healed uneventfully and in the left eye no anatomical deformities were noticed in the lower eyelid and the eye looked normal. No relapse was observed in re-examinations within 18 months after the surgery (Fig. 6). Also no metastases were recorded, local or distant, during the follow-up period.

DISCUSSION

Meibomian gland neoplasms can be categorized histologically as adenomas, ductal adenomas, epitheliomas and carcinomas (Goldschmidt and Goldschmidt, 2017). All have a similar clinical appearance as tan, pink, gray, or black masses extending from the meibomian gland orifice and occasionally can erupt the palpebral conjunctiva (Labelle and Labelle, 2013). Meibomian gland adenomas and adenocarcinomas, according to two retrospective studies, have been reported as the most frequent tumors of the meibomian glands, representing 44%-62% of the canine eyelid tumors in middle-aged or older dogs (Krehbiel and Langham, 1975; Roberts et al., 1986).

Regarding the gross appearance of benign meibomian gland tumors, they present as focal or multifocal nodular masses which are often exophytic and papillary (Dubielzig et al., 2010). Histologically, meibomian gland adenomas have a multilobular arrangement with large vacuolated cells in the center and smaller reserve cells at the periphery of each lobule. Meibomian gland adenocarcinomas appear similar to benign adenomas grossly and microscopically, but adenocarcinomas have a greater percentage of proliferative reserve cells and fewer large foam cells. Also, hemorrhage from hypervascularization and pigmentation from proliferative melanocytes are a common characteristic (Riss et al., 2002). On the other hand, meibomian gland epitheliomas are made up of undifferentiated basal cells with rare sebaceous or squamous differentiation (Dubielzig et al., 2010). Furthermore, in sebaceous epitheliomas mitoses are found only in the reserve cells and do not involve the sebocytes (Goldschmidt and Goldschmidt, 2017). In all benign meibomian gland tumors, a lipogranuloma may be surrounding the tumor (Dubielzig et al., 2010). In the present study, histological examination revealed that both removed masses had sheets of undifferentiated basal cells with rare sebaceous differentiation, focally scattered melanocytes, and mitoses involving the reserve cells, as previously described in the literature.

Until now, objective criteria to differentiate meibomian adenomas and epitheliomas, or the immunohistochemical profile of meibomian gland epitheliomas have not been clearly established (Labelle and Labelle, 2013). According to the literature, epitheliomas should be composed predominantly of basal cells, and some suggest that 90% of the cells must be of the basal type to warrant the diagnosis of epithelioma (Gross et al., 2005). Despite the fact that numerous mitoses may be observed, other features of malignancy are not found in meibomian epitheliomas, therefore some authors classify epitheliomas as benign meibomian adenomas (Labelle and Labelle, 2013).

In dogs, true meibomian carcinomas do occur but are a rare malignant variant of the meibomian gland

tumor (Dubielzig et al., 2010). They are locally invasive and presented with tumor cells with varying amounts of intracytoplasmic lipid vacuoles, anaplastic cellular features and rare meibomian gland secretory features (Dubielzig et al., 2010; Labelle and Labelle, 2013). The nuclei are large and hyperchromatic, with prominent nucleoli, and show pleomorphism and mitotic activity (Labelle and Labelle, 2013). Squamous cell carcinoma affects the non-pigmented eyelids, and is more often in cats, horses and cattle compared to dogs. The preneoplastic lesion appears as an erythematous, scaly area or slightly raised, progressing to an ulcer covered by fibropurulent exudates (Riss et al., 2002). In cats usually it extends into the haired skin, with a concurrent diffuse conjunctival disease (Dubielzig et al., 2010).

Two cases of unilateral meibomian gland epithelioma have been reported in the veterinary literature (Saberi et al. 2012; Choi et al. 2013). Choi et al. (2013) described surgical management of a meibomian gland epithelioma in a thoroughbred horse. Histopathological analysis revealed neoplastic basaloid cells forming irregular cell masses, with mildly pleomorphic and undifferentiated appearances, prominent oval nuclei and scant cytoplasm (Choi et al., 2013). Saberi et al. (2012) reported an 8-year old Doberman that was presented with an enlargement of the upper left eyelid. The mass was removed surgically by exenteration of the globe. Histopathologically, the mass was composed of a solid growth of irregular islands and trabeculae of basaloid reserve cells, interspersed with fewer sebocytes. Based on clinical and histopathological findings, meibomian gland epithelioma was diagnosed. In that case report no follow-up was obtainable as the dog died of unrelated causes (Saberi et al., 2012). In the present case this is the first documented case of bilateral meibomian gland epithelioma in a dog, with no relapse observed within 18 months.

Early surgical removal of any eyelid tumor is the treatment of choice (Frans and van der Woerdt, 2013), with the preservation of the structural and functional integrity of the eyelids being of major consideration. Corneal exposure, irritation, and ulceration may result if the structure and function of the eyelid is altered. Removal is also indicated when eyelid masses are rapidly increasing in size, are ulcerated, or cause corneal irritation (Aquino, 2007). Management of canine eyelid tumors include surgical excision, laser ablation, cryosurgery or combination of the above (Frans and van der Woerdt, 2013). The size and site of the mass, and the involvement of the lid margin will determine the selection of the appropriate surgical technique (Aquino, 2007). Regarding the surgical excision of eyelid masses, if the defect after the excision is less than one-third to one-fourth of the total eyelid length, apposition of the surgical sites can be achieved by sutures. If the lid defect approximates up to one-third of the lid length, a 'relief' lateral canthotomy may decrease the resulting lid tension. The surgical techniques that do not required grafts of adjacent tissues, include the partial-thickness excision, the simple 'V' technique (full-thickness excision), and the four-sided method. In cases that the defect is one-third or more of the length of the eyelid, reconstructive blepharoplastic procedures are required that can be modified for each patient. Some of these techniques include the sliding skin flap, sliding 'Z' skin flap, semicircular skin graft, pedicle skin graft, tarsoconjunctival graft, palpebral conjunctival graft (sliding and free), buccal mucosa grafts, rhomboid grafts, and the 'bucket handle' (Cutler–Beard) procedure (Gelatt and Whitley, 2011).

Meibomian tumors are managed by excision, cryosurgery, or a combination of both (Holmberg, 1980). Recurrence rates between dogs treated with cryosurgery and those treated surgically were not significantly different (15.1% and 10.5%, respectively). The mean recurrence time after cryosurgery was 7.4 months and 28.3 months after surgical excision (Holmberg, 1980; Collier and Collins, 1994), whereas the overall cosmetic appearance was better with cryosurgery (Roberts et al., 1986). Cryosurgery may also be considered in cases where anesthetic risk is high, of recurring tumors, and for surgically difficult cases (Holmberg, 1980). In the present case, exenteration of the right eye was the only option, due to the progression of the tumor to the peribulbar space. Surgical excision was preferred for the mass of the left eye due to the location of the tumor, and the lack of invasion to the periorbital tissues and the globe. The cosmetic appearance for both surgeries was acceptable and no relapse was noticed during the follow-up period.

This report shows that despite the fact that meibomian gland epithelioma is rarely encountered, in relation to meibomian gland adenoma; it should be included in

the differential diagnosis of eyelid tumors. Adenomas and epitheliomas of meibomian glands represent 10% of tumor submissions to Comparative Ocular Pathology Laboratory of Wisconsin. The frequency of both adenomas and epitheliomas of the meibomian glands is thought to be under-estimated since small masses are not usually submitted for histological analysis when excised or treated in such a way that the tissue is destroyed (Dubielzig et al., 2010). Prompt surgical intervention and extended excision is the treatment of choice, even though these tumors show low malignancy. Preservation of the eye globe and vision can be attained in most of the cases.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare for this manuscript.

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Case report
Κλινικό περιστατικό

A case of Amidostomosis in a racing pigeon *(Columba livia)* in Greece

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Περιστατικό αμιδοστόμωσης σε ταχυδρομικό περιστέρι *(Columba livia)* στην Ελλάδα

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ABSTRACT. A dead pigeon (Columba livia) was submitted to the Unit of Avian Medicine, Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. It derived from a pigeon flock consisted of approximately 100 racing birds of various ages between 4 months to 5 years old. The flock was kept on a terrace indoors in 4 cages of 3 m^2 each. Near this flock, there were also two other pigeon flocks in a distance of 20 and 30 meters, respectively. The pigeons' health problem had been present in this flock for approximately 3 years before this delivery. Lack of appetite, poor growth of young birds, weakness, depression, vomiting, diarrhea and emaciation were the most important clinical signs of the pigeons in the flock. During necropsy, discoloration and atrophy of liver, spleen and kidneys, edema of gizzard, necrosis in the koilin layer of the gizzard and under this, presence of small parasites, dilatation in the anterior

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small intestine (duodenum, jejunum) and finally hemorrhagic content in the second half of the small intestine and the rectum were observed. According to the findings of the postmortem and laboratory examination, amidostomosis was determined to be the cause of the pigeon's death. Although pigeon infection by *Amidostomum* spp. is reported around the world, so far, amidostomosis has not been reported in pigeons in Greece. Pigeon owners should take all the appropriate treating and managing measures to control the spread of this parasite and its consequences on their flocks.

Keywords: Amidostomosis, pigeons, gizzard, Greece

ΠΕΡΙΛΗΨΗ. Αγωνιστικό περιστέρι *(Columba livia)* ηλικίας 2 ετών προσκομίστηκε για νεκροτομική εξέταση στη μονάδα Παθολογίας Πτηνών του τμήματος Κτηνιατρικής, ΑΠΘ. Το περιστέρι προερχόταν από ένα σμήνος 100 περιστεριών αγώνων, ηλικίας από 4 μηνών μέχρι 5 ετών. Ο περιστερώνας ήταν τοποθετημένος στην ταράτσα, σε κτίσμα που περιελάμβανε 4 κλουβιά των 3m^2 το καθένα, ενώ σε απόσταση 20 και 30 μέτρων, αντίστοιχα, υπήρχαν ακόμη δυο περιστερώνες. Με βάση τα στοιχεία του ιστορικού, η νοσολογική οντότητα πρωτοεμφανίστηκε στο σμήνος 3 χρόνια πριν την προσκόμιση του συγκεκριμένου περιστεριού. Η συμπτωματολογία της νόσου περιελάμβανε κυρίως συμπτώματα από το πεπτικό, όπως ανορεξία, καθυστέρηση της ανάπτυξης στα νεαρά πτηνά, αδυναμία, έμετοι, διάρροια και αφυδάτωση. Κατά την νεκροτομική εξέταση, παρατηρήθηκαν αποχρωματισμός και ατροφία του ήπατος, της σπλήνας και των νεφρών, οίδημα και αλλοιώσεις του μυώδους στομάχου, παρουσία παρασίτων κάτω από τον επιδερμικό χιτώνα του μυώδους στομάχου, οίδημα στη πρόσθια μοίρα του λεπτού εντέρου (δωδεκαδάκτυλο και νήστιδα) και τέλος, αιμορραγικό περιεχόμενο στο δεύτερο μισό του λεπτού εντέρου και του απευθυσμένου. Η παρασίτωση από *Amidostomum* spp. τέθηκε ως αιτιολογική διάγνωση σύμφωνα με τα νεκροτομικά και εργαστηριακά ευρήματα, και αποτελεί την πρώτη αναφορά για την Ελλάδα. Οι ιδιοκτήτες περιστεριών οφείλουν να λάβουν τα απαραίτητα μέτρα θεραπείας και ελέγχου για να περιορίσουν την ενδεχόμενη επέκταση της νόσου και τις πιθανές επιπτώσεις της.

Λέξεις ευρετηρίασης: Αμιδοστόμωση, περιστέρια, μυώδης στόμαχος, Ελλάδα

INTRODUCTION

Amidostomosis is a chronic parasitic disease of the gizzard in birds (Haralampidis, 2003). The disease is mainly associated with waterfowls (Kavetska et al., 2015), such as geese, ducks and swans (Papazahariadou et al., 1994; Tuggle and Friend, 1999; Papazahariadou et al., 2008). However, the relevant parasites have also been secluded from other species of birds, such as pigeons, chicken (experimental infection) and many other migratory birds (Bowman, 1999; Cole and Friend, 1999; Saif et al., 2008). Mammals are not infected (Bowman, 1999).

The nematodes of the genus *Amidostomum* spp. are the causative agent of amidostomosis. In Europe, there are six common species of this genus i.e. *Amidostomum acutum, A. anseri, A. cygni, A. fulicae, A. henryi, A. spatulatum* (Tuggle and Friend, 1999). There are also other species, such as *A.boschalis* (Haralampidis, 2003). These parasites infect th e upper alimentary tract of the birds, particularly, the solid layer of a carbohydrate-protein complex called the koilin layer of the gizzard and less frequently the proventriculus (S. van Riper and C. van Riper, 1985; Taylor et al., 2007).

The life cycle is similar among the *Amidostomum* species (Saif et al., 2008). More precisely, it is direct, which means that the infective parasitic larva (L3) invades a single host animal and it develops until its reproductive maturity (Tuggle and Friend, 1999). The infection takes place when the bird ingests the L3 larva which is found in the surface of greens and in ponds or when the L3 larva invades the skin of the bird during its swimming. Subsequently, in the first case the infective larva reaches the gizzard through the alimentary tract, whilst in the second case migration of the L3 larva takes place via the lungs and the liver. The larva reaches in reproductive maturity under the gizzard stratum corneum in approximately 15-28 days (in chicks) or in up to 33 days (in adult birds). The parasitic eggs, which are already embryonated are shed through the feces in the outer environment. Subsequently, after its formation,

the L1 larva develops to L3 larva inside the egg in 2-6 days (in 16-27oC) and after that the egg hatches and the L3 larva comes to the outer environment (Haralampidis, 2003; Taylor et al., 2007).

The infective larva survives in the environment for 30 days (in 25oC) or even for 3 months (in 2oC) (Haralampidis, 2003). What is more, it can swim actively in water of ponds for up to 30 days (in 5-10oC) and it is able to reach the surface from depths of up to 10cm (Enigk and DeyHazra, 1970).

During the life cycle, the *Amidostomum* spp, attack the koilin layer of the gizzard of their hosts' (Macklin, 2013). Particularly, the migration and the development of L3 larva as well as the feeding of the blood-sucking adult worm (0.1-0.4 ml blood/adult A. anseris/day) lead to hemorrhage in gizzard and severe erosions of gizzard lining (Beynon et al., 1996; Tuggle and Friend, 1999;Haralampidis, 2003). Large amounts of worms (greater than 35) may denude the whole surface lining of the gizzard, making the edges of the grinding pads degenerate and finally separate from the underlying tissue (Tuggle and Friend, 1999). In addition, enteritis is quite possible to happen, too (Beynon et al., 1996).

There are no pathognomonic signs of αmidostomosis in birds, indicating the gizzard parasitism. However, in cases of heavy infection of young birds some symptoms may appear, such as poor growth, loss of appetite, dullness, emaciation, diarrhea, anemia due to blood loss, change of expected liver seize and even considerable mortality (Kobulej, 1983; Beynon et al., 1996; Tuggle and Friend, 1999; Jordan et al., 2001; Saif et al, 2008).

In other words, the heavy parasitism can induce a chronic debilitating disease and the weakness can be so intense that the aquatic birds cannot hold their neck above water (MacNeil, 1970). Furthermore, birds of all ages may be subject to emaciation and general weakness, too (Tuggle and Friend, 1999). However, according to Herman and Wehr (1954), A. anseris itself is not a primary source of loss but rather an important contributing factor (van Riper and van Riper, 1985).

Consequently, amidostomosis may be an important risk factor in some countries and it should not be neglected (Kobulej, 1983).

CASE HISTORY

This report describes a case of amidostomosis in a pigeon derived from a pigeon flock, which was located in Neapoli, a region of the western part of Thessaloniki. The pigeon *(Columba livia)* was submitted for necropsy to the Unit of Avian Medicine, Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. It was a male bird aging 3 years and hatched in this flock. The flock consisted of approximately 100 racing pigeons of various ages from 4 months to 5 years old. These birds were placed on a terrace indoors, in 4 big cages of $3m^2$ each. Near this flock, there were also 2 other pigeon flocks in a distance of 20 meters and 30 meters respectively. An adequate ration special for racing pigeons as well as fresh water were provided to these birds twice per day. Moreover, cleaning of their environment used to take place on a daily basis. What is more, during autumn 2014 these pigeons participated in racing competitions and the last vaccination against *Paramyxovirus* took place a year before the submission of the dead pigeon to the clinic of Avian Medicine. The pigeons' health problem had been present in this flock for approximately 3 years before this submission. The symptoms manifested in this flock were not typical of any particular disease. Lack of appetite, poor growth of young birds, weakness, vomiting, diarrhea and emaciation were the most important of them. Furthermore, it should be mentioned that 30 birds had died the previous month.

Post mortem examination of the pigeon revealed discoloration and atrophy of the liver, spleen and kidneys, edema of the gizzard, necrosis in the koilin layer of the gizzard and under this, presence of small parasites. Furthermore, dilatation was also observed in the anterior small intestine (duodenum, jejunum), whilst the second half of small intestine and the rectum were full of hemorrhagic content. Macroscopic lesions were not found in other organs, while the bacteriological examination of liver and spleen was negative. Direct microscopy of smears was negative for *Histomonas* spp.

The parasites and the relative infected tissues were transferred to the Laboratory of Parasitology, Veterinary Faculty of Aristotle University of Thessaloniki for parasite recovery. The nematodes

were collected, cleaned with saline and identified under the stereoscope using morphological identification keys provided by Taylor et al (2007). The worms were identified to be *Amidostomum* spp. According to the findings of the postmortem and laboratory examination, amidostomosis was determined to be the cause of the pigeon's death.

DISCUSSION

Amidostomosis is arguably a common infection in birds, such as geese (Kobulej, 1983), ducks (Borgsteede, 2005) and other aquatic birds (Taylor et al., 2007). In Greece the parasite has been found in swans (Papazahariadou et al., 1994), geese and ducks (Papazahariadou et al. 2008), but not in pigeons, so far. Therefore, taking into account 1) that the infection by *Amidostomum* spp. is more common in aquatic birds and 2) that the pigeon examined derived from a racing pigeons flock in a terrace potentially in contact with aquatic birds which travel to the lakes around the region of Thessaloniki, it is readily understood that this contact maybe a major risk factor of this case of pigeon amidostomosis.

In addition, seasonality is not normally associated with this parasitism in migratory aquatic birds, because these birds become firstly exposed on breeding grounds and then they continue to be exposed throughout their lives (Tuggle and Friend, 1999). However, the problems of this pigeon flock and the case of the pigeon amidostomosis happened during autumn, which is the main period of migration for many aquatic species. In other words, this is also the most possible period for the contact between pigeons and wild aquatic birds. Thus, migration period of aquatic birds could be another possible risk factor of our amidostomosis case.

In order to deal with this case of pigeon amidostomosis, both chemotherapeutics and managing practices should be taken. The treatment of clinical or suspected subclinical cases of amidostomosis can be based on medical substances, such as cambendazole (60 mg/kg) against adult worms and larvae, pyrantel (100 mg/kg) against adult worms, citarin (40mg/kg), mebendazole (10mg/kg) and fenbendazole. However, toxicity has been reported in pigeons that received fenbendazole per os at the rate of 30 mg/kg for 5 days. Furthermore, flubendazole and ivermectin have been proven to be effective against this parasitic infection as well as albendazole and piperazin (Baker, 2007; Islam et al. 2012; Macklin, 2013).

However, apart from chemotherapeutic treatment, the control of amidostomosis depends on managing practices, as well. Particularly, these practices should focus on the disruption of the parasite's life cycle. In detail, the possibility of the parasite transmission is greater in crowded and continuously used habitat, because the combination of accumulative fecal contamination and warm ambient temperatures (20-25o C) promote the quick larval development. Additionally, newly hatched birds are least resistant to infection and birds of all ages are susceptible to reinfection. Hence, proper sanitation and good managing practices are of the utmost importance (Tuggle and Friend, 1999; Baker, 2007).

Although, human health considerations have not been reported so far, people who eat gizzards of relative birds (including pigeons) should cook them thoroughly, discard pigeons' appearing relative clinical signs or lesions and take all the appropriate treating and managing measures to control the prevalence of the parasite and its consequences in their flocks (Tuggle and Friend, 1999).

Although *Amidostomum* spp. has been reported in aquatic birds in Greece, this is the first case of *Amidostomum* spp. in racing pigeons. Therefore, further epidemiological studies are needed, in order to estimate its prevalence in pigeon flocks in different regions of Greece and to elucidate the route of transmission to pigeons.

CONFLICT OF INTEREST

The authors of this paper certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

ETHICAL CONSIDERATIONS

On behalf of all authors it is certified that legal and ethical requirements have been met with regards to the humane treatment of animals described in the study.

Figure 1: Edema of the gizzard, necrosis in the koilin layer and presence of numerous parasites in a racing pigeon *(Columba livia)*.

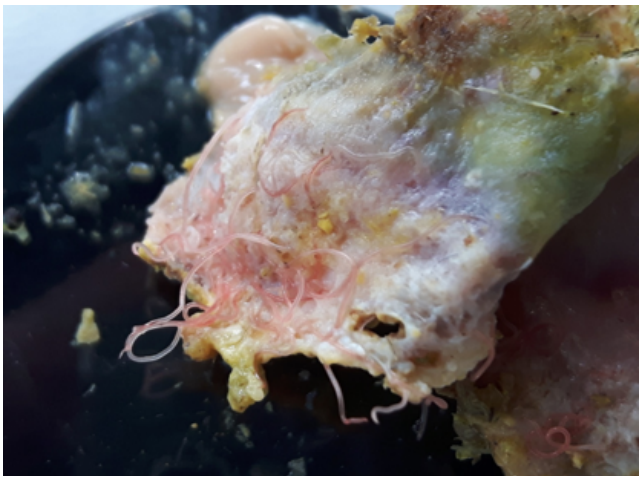
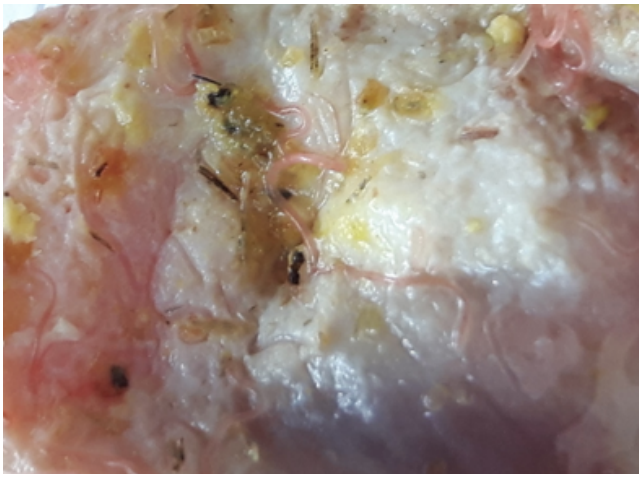


Figure 2. Necrosis and presence of numerous parasites under the koilin layer of the gizzard in a racing pigeon *(Columba livia)*.



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Case report
Κλινικό περιστατικό

Perivulvar squamous cell carcinoma in a cow

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ABSTRACT. We present a case of perivulvar squamous cell carcinoma in an 8-year-old crossbred Simmental cow. A tumoral mass, of considerably large volume localized in the perivulvar region and growing at a slow pace, was detected in the animal. The mass, subsequently identified histopathologically and immunohistochemically as squamous cell carcinoma, was surgically excised from the perivulvar region using intrathecal anesthesia.

Keywords: cow, spinal anesthesia, vulva, squamous cell carcinoma.

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INTRODUCTION

Vaginal malignant tumors are uncommon; however, recurring squamous cell carcinomas in the vulva are frequently encountered (Khodakaram-Tafti et al., 2013). Squamous cell carcinomas originating from stratum spinosum cells are mainly seen in adult cows, sheep, and mares. While this tumor is observed in various parts of the body, it is typically located in non-pigmented and hairless regions. Prolonged exposure to ultraviolet rays is considered an important factor related to tumor formation (Erer and Kıran, 2005; Hillman and Gilbert, 2008; Khodakaram-Tafti et al., 2013; McEntee, 1990; Pimenta-Oliveira, 2011). Squamous cell carcinoma, which has an aggressive character, may infiltrate locally or metastasize if not diagnosed in the early stage (Hillman and Gilbert, 2008). Clinically, it may have a productive or erosive character. The productive type may reportedly be cauliflower-like in appearance, and there may be bleeding and ulceration present on the surface (Agnew and MacLachlan, 1990; Erer and Kıran, 2005; McEntee, 1990).

Squamous cell carcinoma of the vulva has typically been reported in cattle, sheep, goats, and sometimes other mammalian species (Khodakaram-Tafti et al., 2013; McEntee, 1990). Tumors of vaginal origin are detected particularly during colposcopy or artificial insemination procedures (Hillman and Gilbert, 2008; Meyers and Read, 1990). Squamous cell carcinoma was observed in 0.44% of 7483 cattle during necropsy, and in 4.97% of tumor-detected cases (Rosa et al., 2012). Yeruham et al., (1999) reported vulvar squamous cell carcinoma detection at a rate of 0.91% in dairy cattle and 0.38% in beef cattle. In Turkey there are a limited number of reports regarding vulvar squamous cell carcinoma in cattle (Alaçam et al., 1981; Öcal et al., 1995).

Early diagnosis increases the possibility of success in the treatment of squamous cell carcinoma of the vulva. In such cases, the treatment of choice is cryotherapy and wide surgical excision (Hillman and Gilbert, 2008; McEntee, 1990).

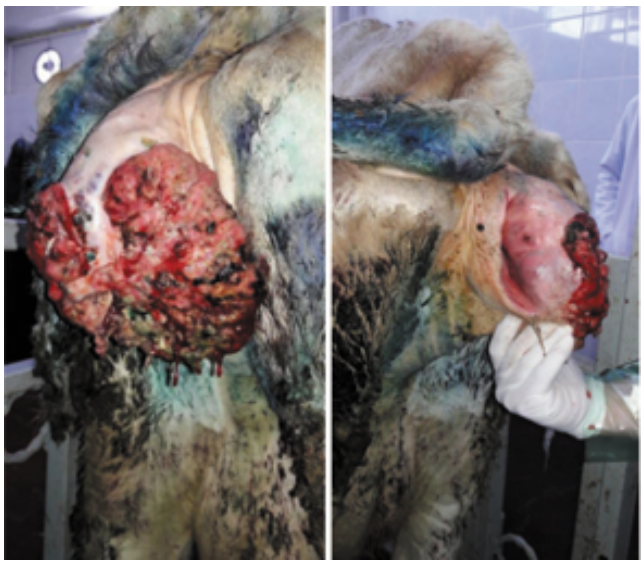
Here we aimed to present pathological findings of perivulvar squamous cell carcinoma diagnosed in a crossbred Simmental cow and to describe surgical removal of the tumor using intrathecal anesthesia.

CASE HISTORY

The present case involves an 8-year-old crossbred Simmental cow which had given birth for the fifth time one month prior. The clinical history revealed that the cow had a normal delivery during which a mass with a diameter approximately 3-4 cm was noticed on the right side of the vulva. The mass had increased within 1 month following the delivery (roughly 10 cm) and would occasionally bleed.

During a clinical examination of the animal by the Obstetrics and Gynecology Clinic of Kafkas University Veterinary Faculty, a bleeding and necrotic in some regions, ulcerated multi-nodular mass was detected on the right side of the vulva (Figure 1). No diseases or symptoms other than the mass were observed. Upon the rectal palpation, the uterus and ovaries were determined to be normal. Based on the clinical evaluation, a decision was made to remove the mass surgically using the spinal (intrathecal) anesthesia Bupivacaine (0.5%, Marcaine®, 5 mg/mL, Astra Zenaca) via a 20 gauge spinal needle for subdural injection.

Figure 1: Large tumor mass on the vulva with ulcer, bleeding, and necrosis.



The animal was placed on a Hannover carriage in the right lateral position and immobilized appropriately to prevent cranial extension of the anesthetic agent during injection. After shaving and antisepsis of the lumbosacral region, a spinal needle was extended vertically until it passed the

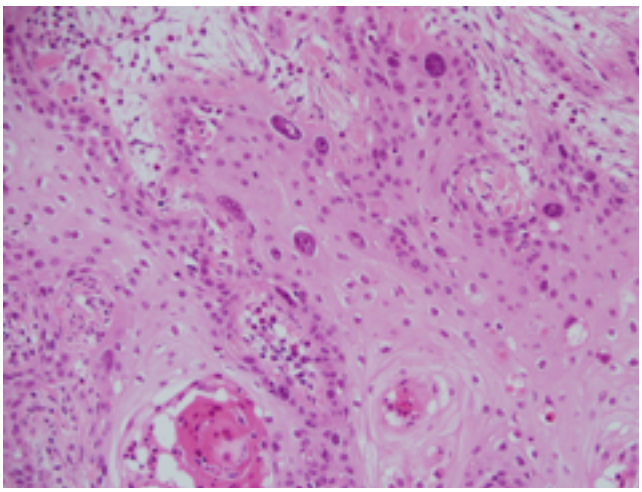
ligamentum flavum through the lumbosacral space. After noting that it touched the dura mater, the needle was pushed slightly forward to enter the subdural space. The flow of cerebrospinal fluid (CSF) was observed by removing the stylet from the spinal needle. A certain amount of CSF was aspirated to avoid a pressure increase as a result of the local anesthetic to be injected. A volume of 10 mL of bupivacaine, previously loaded into an injector, was slowly introduced into the spinal cord. Once anesthesia of the tail and vulva area was achieved, the mass was removed from the perivulvar area following a routine surgical procedure with bleeding controlled by electrocautery. Routine parenteral fluid infusion (Lactated Ringers) throughout the operation and postoperative antibiotic administration was performed.

The excised mass was measured 15x10x20 cm and exhibited ulcers and bleeding at the surface. When the mass was cross-sectioned, multiple small and hard greyish nodules with a cauliflower appearance on the cut surface were noticed. Since the animal's owner rejected euthanasia, an examination of both carcass and regional lymph nodes could not be performed, and therefore the presence of metastasis in internal organs could not be evaluated.

The mass was sent to the Department of Pathology for histopathological examination. Tissue samples taken from the mass were fixed in 10% buffered formalin solution. Routinely prepared paraffin blocks were cut to 5 μm thickness, stained with hematoxylin and eosin, and evaluated by light microscopy. For immunoperoxidase staining, serial sections 4 um in thickness were deparaffinized in xylene and hydrated through grading alcohols. The sections were incubated in 3% H_2O_2 for 20 min at room temperature to block endogenous peroxidase activity. After the sections were washed for 5 min in Tris buffered saline (TBS), they were boiled in citrate buffer saline (pH 6.0) for 20 min in a microwave oven to induce antigen release. All sections were stained with Genemed Acu-Stain Mouse+Rabbit HRP Instant Kits using mouse monoclonal Ki-67 (Genemed, Clone; GM010), mouse monoclonal anti-p53 (Biorbyt, Clone; SPM590) and mouse monoclonal anti-cytokeratin (Thermo Scientific, Clone; DE-SQ) primer antibodies according to the manufacturers' instructions. For immunolabeling, 3,3'-diaminobenzidine (DAB) was used as the chromogen. Mayer's hematoxylin was used as the counterstain. Negative control sections were incubated with TBS instead of the primer antibodies.

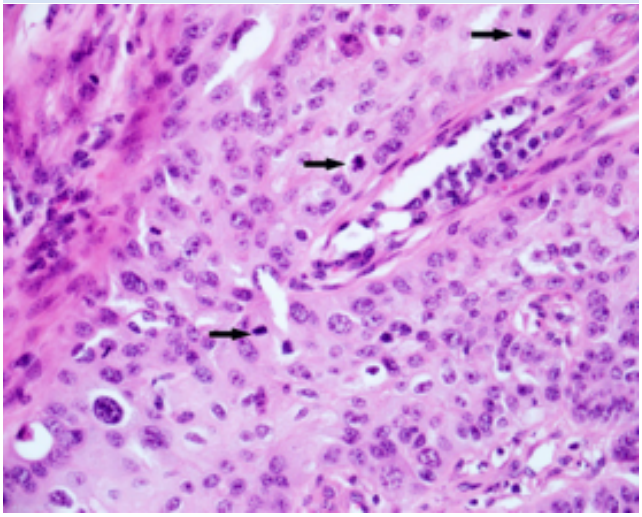
Histopathologically, the tumor mass was diagnosed as squamous cell carcinoma. Neoplastic cells showed often nuclear pleomorphism. The cells had an eosinophilic cytoplasm with considerably large, hyperchromatic, and ovoid nuclei, with at least 2-3 nucleoli. Neoplastic proliferation revealed frequently keratin pearls consisting of laminated keratin structures (Figure 2). Keratin pearls were not seen in all of the tumor islands, and a small number of tumor cells showed immature keratinization within the cytoplasm. In particular, in some areas close to the surface of the mass, diffuse and severe neutrophilic infiltration along with bacterial clusters, necrosis, and thrombi were observed. Interestingly, eosinophil leukocyte infiltrations were commonly seen in the mass. Pleomorphic tumor cells showed frequent mitotic figures numbering 7-10 on a microscopic field of high magnification (40x) (Figure 3). Tumor cells spread into the dermis in some areas, and in some regions of the mass, neoplastic proliferations were supported by delicate stromal tissue.

Figure 2: Characteristic keratin pearl in tumor tissue and neoplastic cells with considerably large nuclei, (H&E x 20).



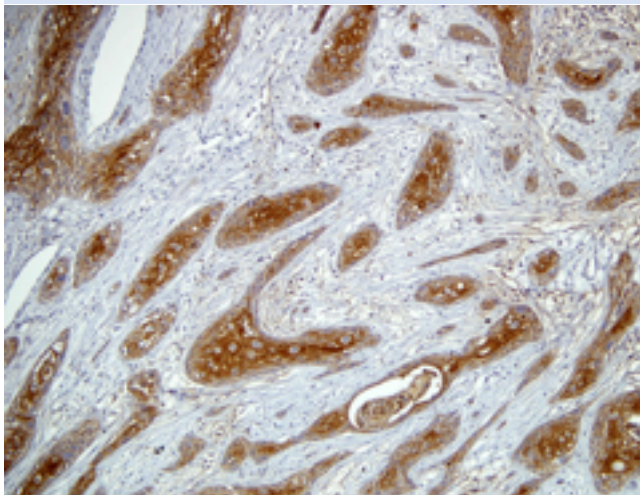
Immunohistochemically, a severe cytoplasmic positive reaction of neoplastic cells for cytokeratin was observed in the entire tumor tissue. But there was

Figure 3: Mitotic figures (arrows) in the tumor tissue (H&E x40), (arrows)(H&E4.)



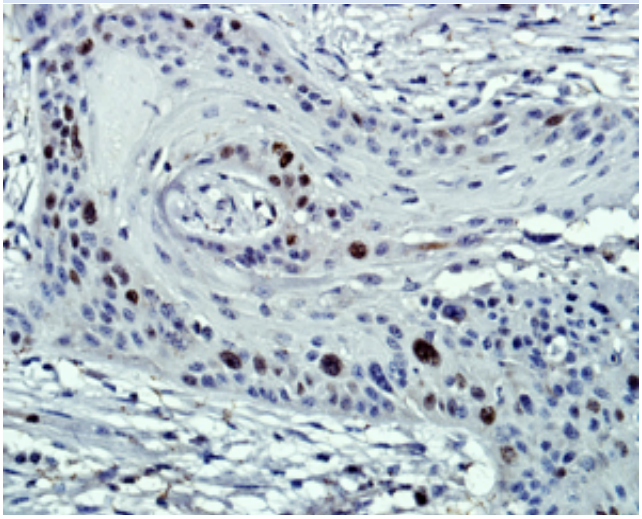
no reaction in the stromal tissue. (Figure 4). Immune reaction products appeared to be fine granular and more intense in the areas adjacent to the cellular

Figure 4: Intense cytoplasmic positive reaction (ABCx10).



membrane, with no staining of desmosomes. In addition to the tumor cells, a cytokeratin-positive reaction was also detected in the keratin pearls. Ki-67 staining revealed tumor cells with nuclear positive labelling. Positive staining was also found in the nuclei of hyperchromatic cells and in cells undergoing mitosis (Figure 5). In the large neoplastic cells, nuclear staining spread to the whole nuclei in the form of particles. Additionally, Ki-67 nuclear-positive labelling was observed in a small number of germinative cells of the epidermal layer.

Figure 5: Ki67 nuclear positive reaction in tumor cells (ABC x 40)



DISCUSSION

Tumors such as squamous cell tumors, leiomyoma, fibroma, fibro-papilloma, hemangioma, fibrosarcoma, leiomyosarcoma, and melanoma can be located in the vulva and vagina of cattle (Kuru et al., 2016; Yeruham et al., 1999). Squamous cell tumors can be found especially in pigment-free regions of the body, and are more frequently detected in elderly animals (Pandey, et al., 2010; Prasath et al., 2009). In the case presented, it was observed that the mass was only on the right side of the vulva.

Vaginal tumors are typically excised by electrocautery. Small-scale masses can easily be removed with sedation and local anesthesia, while removal of large masses requires more substantial anesthesia (Çolak et al., 1997; Enginler et al., 2011; Kuru et al., 2016). Lower or upper epidural anesthesia is generally preferred for large-scale gynecologic operations in cattle (Hillman and Gilbert, 2008). In this case, the surgical operation was performed with intrathecal anesthesia, and no signs of pain or anesthetic complications were encountered during the intra-operative period.

Khodakaram-Tafti et al., (2013) reported that squamous cell carcinoma produces keratin pearls and has a good level of differentiation, and that this tumor also metastasizes to the nearest lymph node nodules. In the present case, we detected numerous bleeding and necrotic masses; however, the clinical examination revealed the lymph nodes (prefemoral, prescapular

and supramammary) to be of normal size and surface. This situation may be due to the relatively short tumor history. In the literature (Khodakaram-Tafti et al., 2013; Yeruham et al., 1999), it has been suggested that squamous cell carcinomas are highly invasive and metastasize to the lymph nodes. However, since postmortem examination could not be performed on the present case, it was not possible to determine whether the tumor had metastasized to surrounding tissues and organs.

Histopathological and immunohistochemical studies revealed that the microscopic findings generally corresponded with the literature data, and the determination that the tumor was a squamous cell carcinoma. Epithelial proliferative foci and keratin pearls in the form of submucosal islets and cords in the neoplastic epithelial cells of this squamous cell carcinoma have also been described by other authors (Devi et al., 2010; Pimenta-Oliveira et al., 2011). Likewise, keratinization is generally seen in tumor cell foci and in individual cells. The formation of loose connective tissue which were widely observed in the tissue of the tumor have been described as desmoplasia/stromal fibroplasia in the literature and it has been suggested that they are important in the diagnosis of invasive carcinomas. The tumor parenchyma supported by fibrotic tissue in the present case supports this theory. Ulceration, necrosis, and inflammatory cell infiltration that are commonly seen in the tumoral tissue are explained by ulceration and contamination of the surface of the mass (Devi et al., 2010; Khodakaram-Tafti et al., 2013; Pimenta-Oliveira et al., 2011). In the presented case, tumor tissue was stained using the immunoperoxidase technique with cytokeratin and Ki67 markers. The cytokeratins are considered to be the largest intermediate filaments of squamous epithelium and are critically important for the stabilization, shape, intracellular communication and transport within the cell. Therefore, cytokeratin expression is of considerable importance in tumor progression (Frohwitter et al., 2016). Based on cytokeratin immunostaining, all of the tumor parenchyma and squamous mucosal epithelial cells were identified as having positive cytoplasmic staining, and the tumor was confirmed to be of epithelial origin. Similarly, in some studies (Devi et al., 2010; Vala et al., 2001), pervasive positive cytokeratin staining of tumor cells has been observed for ocular squamous cell carcinomas in cattle. The importance of cytokeratin staining for the identification and differentiation of tumor origins has been emphasized. Neoplastic cells in the tumor tissue were also observed to be nuclear positive for the Ki67 protein. As stated by Pimenta-Oliveira et al. (2011), frequent mitoses together with nuclear pleomorphism and desmoplasia are indicative of a poor prognosis.

In conclusion, based on characteristic histological features, the neoplasm was diagnosed as a vulvar squamous cell carcinoma. The importance of early diagnosis and treatment is emphasized, as a good prognosis is possible if the mass is surgically removed in the early phase of the disease. In addition, an intrathecal anesthetic protocol can be successfully utilized for the surgical intervention

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Case report
Κλινικό περιστατικό

Ciliary body cysts associated with glaucoma in a Great Dane

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ABSTRACT. An 8-year-old female Great Dane, was referred to the Ophthalmology Unit of the Centre Hospitalier Vétérinaire (CHV) des Cordeliers, for corneal opacity of the left eye that did not respond to topical treatment, administered by the referring vet. On initial examination, bilateral ciliary body cysts were noticed. Lens subluxation, pseudo-plateau iris and glaucoma were observed on the left eye. The diagnosis was confirmed by applanation tonometry and high resolution ocular ultrasonography. Medical treatment resulted in temporary clinical improvement of the left eye. No signs of glaucoma were observed on the right eye upon the last re-check examination.

Keywords: glaucoma, ciliary body cyst, Great Dane.

ΠΕΡΙΛΗΨΗ. Σκύλος οκτώ ετών, θηλυκός ακέραιος, φυλής Great Dane, παραπέμφθηκε στην Οφθαλμολογική Μονάδα του Centre Hospitalier Vétérinaire (CHV) des Cordeliers με συμπτώματα θόλωσης του κερατοειδούς του αριστερού οφθαλμού, μη ανταποκρινόμενη σε τοπική αγωγή, που χορηγήθηκε από τον θεράποντα κτηνίατρο. Κατά την κλινική εξέταση, παρατηρήθηκαν κύστεις του ακτινωτού σώματος αμφοτερόπλευρα. Επιπλέον ο αριστερός οφθαλμός παρουσίαζε υπεξάρθρημα του κρυσταλλοειδούς φακού, ίριδα ψευδο-plateau και γλαύκωμα. Η διάγνωση επιβεβαιώθηκε με μέτρηση της ενδοφθάλμιας πίεσης και υψηλής ευκρίνειας οφθαλμολογικό υπέρηχο. Μετά την έναρξη της φαρμακευτικής αγωγής παρατηρήθηκε παροδική ύφεση των συμπτωμάτων στον αριστερό οφθαλμό, ενώ δεν παρατηρήθηκε εικόνα γλαυκώματος στον δεξιό οφθαλμό έως και κατά την τελευταία επανεξέταση.

Keywords: γλαύκωμα, κύστη ακτινωτού σώματος, Great Dane

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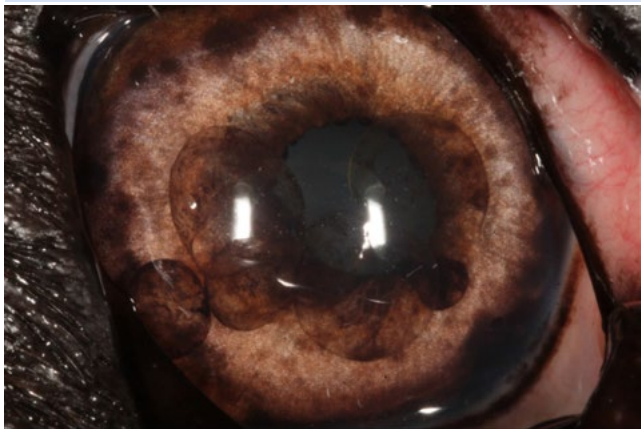
CASE HISTORY

An 8-year-old female Great Dane was referred to the Ophthalmology Unit of the CHV des Cordeliers for corneal opacity of the left eye (OS) evolving over the last month. Topical treatment with framycetine and dexamethasone ointment (Fradexam®, TVM) prescribed by the referring veterinarian did not lead to clinical improvement. No history of uveitis or trauma was reported. An ophthalmic examination was performed. The OS was buphthalmic. Visual loss of the OS was evidenced during the obstacle course. Menace response, dazzle reflex and direct pupillary reflex were negative on the left eye (OS), and positive on the right eye (OD). The indirect pupillary reflex was negative in both eyes (OU). At slit lamp examination (SL-15, Kowa, Torrance, CA, USA) of the OD, multiple ciliary cysts were noticed in the anterior chamber and in the posterior chamber after dilation with tropicamide (Tropicamide®, VIDAL). Iris discoloration was evidenced (Figure 1). Incipient cataract was noticed. The intraocular pressure (IOP), as measured by applanation tonometry (Tono-pen Vet, Medtronic Solan, Jacksonville, FL, USA), was 12 mmHg on the OD, as opposed to 56 mmHg on the OS. The OS presented buphthalmos. At slit lamp examination, conjunctival and episcleral vessels were hyperaemic and tortuous and diffuse endothelial oedema was also seen in the cornea (Figure 2). Multiple ciliary cysts were visualized in the anterior and posterior chamber. Mydriasis and dyscoria were also noted.

Figure 1. Buphthalmos and corneal oedema are noted on the OS. Ciliary body cysts are visualised in the anterior chamber of the OD.

Figure 2. The OD presents multiple ciliary body cysts in the anterior chamber. Note the iris depigmentation.



Lens subluxation was evidenced. The lens was displaced towards the nasal quadrant, without any significant anterior or posterior displacement. Pigment deposition on the anterior capsule of the lens was noticed. Fundus examination with indirect ophthalmoscopy showed media opacity. Gonioscopy (Koeppe pediatric lens) was not easily performed on the OD since it was impeded by the uveal cysts; however, a narrow angle was evidenced.

High resolution ocular ultrasonography (MyLab Sat, Esaote, Genova, Italy, linear 22 MHz probe) was performed on both eyes (OU). The antero-posterior axis measured was 22mm and 26mm on the OD and the OS, respectively. Multiple ciliary cysts, measuring between 2 and 5 mm, were present in the anterior and posterior chamber OU. Cysts located on the level of the iridociliary junction led to anterior displacement of the iris, and consequent angle closure and ciliary cleft collapse (pseudo-plateau iris) on the temporal quadrant of the OS (Figure 3). The OS presented increased corneal thickness and hyper-echogenicity and the anterior chamber was hyper-echogenic. The lens presented cortical hyper-echogenicity. The increased distance between the lens and the ciliary body, the flattened ciliary body and the round silhouette of the lens in the temporal quadrant confirm the zonule rupture and lens subluxation. The vitreous presented hetero-echogenicity and excavation of the optic disc was noted. On the OD, cysts were also located in the posterior segment (Figure 4). Hyper-echogenicity of the anterior lens pole and equator was observed.

Due to the severity and chronicity of glaucoma

Figure 3. US image of the temporal quadrant of the OS. Note the increased corneal thickness and hyperechogenicity, the cysts in the posterior chamber, the iris angulation, the lens subluxation and hyperechogenicity and the hyperechogenic vitreous chamber.

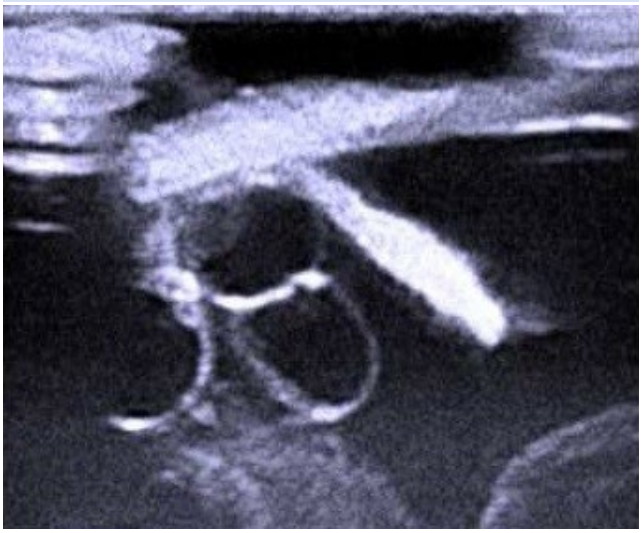
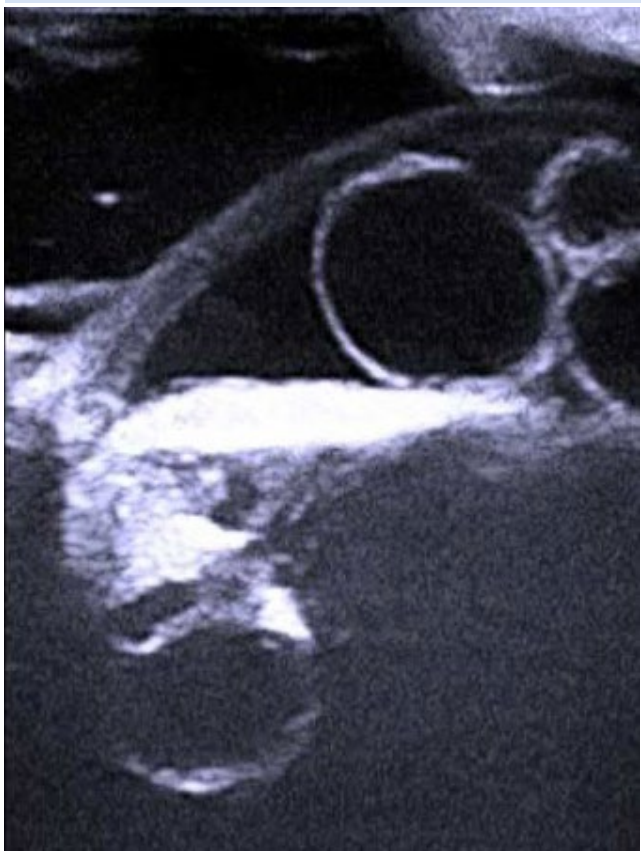


Figure 4. US image of the temporal quadrant of the OD. Multiple cysts are present in the anterior and posterior chamber. Note the presence of one cyst in the posterior segment.



on the OS, the options discussed included intrascleral prosthesis, cyclodestructive procedures, or enucleation. For the OD, needle-aspiration of the cysts and regular follow-ups were suggested. However, the owner did not opt for the surgical recommendations and medical treatment was initiated. Prednisone (Dermipred 20®, VIDAL) at an initial dose of 1 mg/kg PO q 24 h followed by gradual reduction over a three-week period, dorzolamide and timolol ophthalmic solution (Cosopt®, VIDAL) OS TID, travoprost ophthalmic solution (Travatan®, VIDAL) OS BID and retinol and lanoline ophthalmic ointment (Vitamine A Dulcis®, VIDAL) OS BID were prescribed.

Upon the three-week recheck examination, the IOP was 18 mmHg on the OS and 12 mmHg on the OD. The corneal oedema previously reported on the OS had resolved and buphthalmos was less evident. However, vision loss was still evidenced. Prednisone was discontinued. The prescription of local treatment was renewed and a recheck examination in three weeks was recommended.

The patient was re-presented two months later. The OS presented ocular hypertension at 43 mmHg, while the IOP of the OD was of 12 mmHg. Buphthalmos, corneal oedema and vision loss of the OS were noticed. Surgical options were discussed and a date was set for the enucleation of the OS. However, the dog was not presented for surgery on the fixed date.

DISCUSSION

This is the second report in the literature of glaucoma associated with ciliary body cysts in the Great Dane (Spiess et al., 1998). Although uveal cysts are a frequent incidental finding in veterinary ophthalmology, especially in Golden Retrievers, Labrador Retrievers and Boston Terriers (Hendrix, 2013), there are only a few reports of cystic glaucoma. Three breeds have been reported to develop cystic glaucoma: the Golden Retriever (Plummer et al., 2013), the American Bulldog (Pumphrey et al., 2013), and the Great Dane (Spiess et al., 1998). In the Great Dane, glaucoma appears as a result of pseudo-plateau iris, due to mechanical anterior displacement of the iris by multiple ciliary body cysts located in the posterior chamber, and pre-iridal fibrovascular membranes (PIFMs) (Spiess et al., 1998)..

On high-resolution ocular ultrasonography, pseudo-plateau iris configuration was evidenced on the temporal quadrant of the OS. Plateau iris configuration consists of anterior displacement of the peripheral iris, placing it in apposition with the trabecular meshwork and leading to angle narrowing or closure, with a flat iris plane from pupil to periphery and a normal central anterior chamber depth (Stamper et al., 2009). Plateau iris syndrome is defined as the persistence of this configuration following patent iridotomy (Stamper et al., 2009). Pseudo-plateau iris refers to plateau iris configuration that is caused by cysts in the posterior chamber (Stamper et al., 2009). It is among the most probable etiopathogenic mechanisms of cystic glaucoma in the Great Dane. Ciliary body cysts have been associated with plateau iris syndrome and glaucoma in humans (Azuara et al., 1996; Crowston et al., 2005; Le corre et al., 2009; Kitouni et al., 2015). In human medicine, whether or not cysts are inclined to cause angle closure depends on the number, size and location of the cysts. It has been reported that only an angle closure greater than 180° may lead to glaucoma (Ispa-Callen et al., 2009). Moreover, cysts larger than 0.8 mm located at the iridociliary sulcus may also be responsible for angle closure (Wang and Yao, 2012), and among eyes with angle closure, cyst size in patients with multiple cysts was significantly smaller than in patients with a single cyst (Maraone et al., 2014). To the authors' knowledge, there is no data regarding the extent of closure necessary to affect the IOP in dogs.

Pre-iridal fibrovascular membranes (PIFMs) have been associated with glaucoma in human and animal patients (Plummer et al., 2013). They were histologically identified in all eyes of the Spiess et al. study (1998) and were considered another possible etiopathogenic mechanism of glaucoma in association with pseudo-plateau iris. A study showed that in human patients with neovascular glaucoma, the non-pigmented ciliary epithelium has been identified as an essential source of synthesis of vascular endothelial growth factor (VEGF) (Chalam et al. 2014), which is also the predominant vasogenic protein responsible for PIFMs in dogs (Grahn and Peiffer, 2013). Further research would be needed to evaluate the effect of ciliary body cyst formation on the synthesis of VEGF.

Lens subluxation was observed on the OS. In glaucomatous eyes with lens luxation, it may be challenging to precise if glaucoma is primary or secondary to the lens luxation. Various changes associated with lens luxation, such as pupillary block or substantial amounts of prolapsed vitreous obstructing the aqueous outflow and secondary uveitis, may be responsible for the development of glaucoma, and lens luxations are among the most frequent causes of secondary glaucoma in the dog. However, Great Danes are not predisposed to lens luxation (Davidson and Nelms, 2013) and the contralateral lens was not luxated, neither presented instability. Moreover, subluxations, as observed in our case, are more commonly secondary to buphthalmia, due to stretching and eventual rupture, tearing or disinsertion of the ciliary zonules (Plummer et al., 2013). Thus, the lens subluxation on the OS appears to be secondary to glaucoma rather than the primary cause. However, three voluminous cysts were located on the temporal quadrant and may also be responsible for the zonule rupture [a rare complication reported in human literature (Lois et al., 1998)].

Diagnosis of ciliary body cysts in this case report was based on clinical appearance and was confirmed by high-resolution ocular ultrasound. Transillumination of the cysts with a bright light source is another method to differentiate cysts from neoplasms, and was performed for cysts in the anterior chamber. However, high-resolution ocular ultrasound presents higher sensitivity and sensibility (Hendrix, 2013). Ultrasound biomicroscopy has been reported as effective in the diagnosis, size evaluation and localisation of ciliary body cysts in humans (Maraone et al., 2014) and more effective than standard ocular ultrasonography (7 to 12 MHz) in the detection of canine uveal cysts (Taylor et al., 2015); thus, it may consist the method-of-choice for early diagnosis of cyst formation or detection of small-size cysts on the contralateral eye, especially in human patients that usually present smaller-size cysts [0.6547 ± 0.2319 mm (Wang and Yao, 2012), 0.81 ± 0.35 mm (Maraone et al., 2014)]. However, its use may be impractical for animal patients, as heavy sedation or general anaesthesia may be required. To the authors' knowledge, this is the first report that provides ultrasound images for ciliary

body cysts associated with pseudoplateau iris and glaucoma in the Great Dane.

Several treatment options are available depending on the stage of the disease, but they can be divided in three large groups: treatment addressing the cysts, the plateau iris configuration and the glaucoma.

Uveal cysts are usually benign, however, in case of pupil occlusion or angle closure, cyst removal is necessary. Cyst puncture and aspiration of the cyst content resulting in cyst wall collapse can be performed using a small-gauge needle (Hendrix, 2013), especially if few large cysts are responsible for the angle closure (Stamper et al., 2009). Ciliary body cysts are usually not amenable to deflation by semiconductor diode lasers (Hendrix, 2013). However, in human literature, it has been suggested that Nd-YAG laser cystotomy may be effective (Stamper et al., 2009). There is no data in the literature regarding the incidence of glaucoma among Great Danes that present ciliary body cysts. The report of Spiess et al. (1998) and a recent human study (Xue et al., 2017) showed correlation between cysts and glaucoma, and uveal cysts and elevated IOP, respectively, indicating that extended follow-up would be appropriate for these patients and should include IOP monitoring.

Argon laser iridoplasty (Crowston et al., 2005; Ang et al., 2008; Ispa-Callen et al., 2009) and endocycloplasty (ECPL) (Pathak-Ray and Ahmed, 2016) are both techniques that alter the iridocorneal apposition observed in pseudo-plateau iris, and they have been documented as effective in some human patients. However, recurring cysts and secondary angle closure following laser iridoplasty have been reported (Ispa-Callen et al., 2009; Pathak-Ray and Ahmed, 2016), and anatomical differences between dogs and humans, such as Schlemm's canal, may result in different success rates.

Medical glaucoma therapy alone for the narrow- or closed-angle glaucomas is usually ineffective in the long term (Spiess et al., 1998; Hendrix, 2013; Plummer et al., 2013), but, it is often necessary pre- and post-operatively (Plummer et al., 2013), in order to maintain the IOP within the target levels, after pseudo-plateau iris has been addressed. Surgical techniques, such as gonio-implants, trans-scleral laser cyclophotocoagulation or cyclocryothermy, may consist alternative or adjunctive therapeutic choices (Hendrix, 2013).

In summary, although uveal cysts are a common incidental finding, they may be associated with glaucoma in the Great Dane. High-resolution ocular ultrasound is useful in the diagnosis of cysts and pseudo-plateau iris.

CONFLICT OF INTEREST

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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