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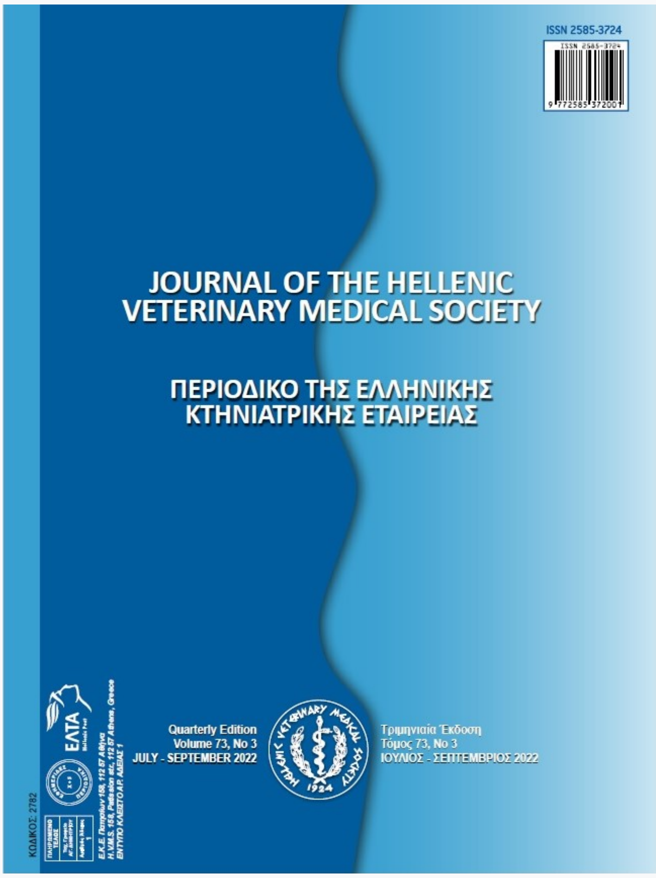

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A Papapostolou, EJ Petridou, K Papageorgiou, A Stournara, A Benos, S Lafi, N Giadinis

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Research article
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Seroprevalence and risk factors assessment of *Brucella* spp. in dairy buffaloes in mainland Greece

A. Papapostolou[1], E.J. Petridou[1], K. Papageorgiou[1], A. Stournara[2], A. Benos[3], S.Q. Lafi[4], N.D. Giadinis[5].

[1] Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

[2] Hellenic Ministry of Rural Development and Food, Department of Veterinary Laboratory, National Reference Laboratory for Brucellosis, Larisa, Greece

[3] Laboratory of Primary Health Care, General Practice and Health Services Research, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece

[4] Department of Epidemiology and Biostatistics, Faculty of Veterinary Medicine, Jordan University of Science and Technology (JUST), Irbid-Jordan

[5] Clinic of Farm Animals, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

ABSTRACT: Brucellosis is an infectious bacterial disease caused by members of the genus *Brucella,* affecting both humans and animals, resulting inserious economic losses in animal production sector as well as deterioration of public health. A cross-sectional study was conducted from January to August of 2018 to determine the seroprevalence and associated risk factors of bovine brucellosis, in Regions of Central Macedonia and Eastern Macedonia and Thrace, North Greece. A total of 1,255 blood samples were collected using a simple random sampling technique from dairy buffaloes older than12 months. All serum samples were analyzed with Rose Bengal Test for screening and Complement Fixation Test for confirmation of the positive samples. Accordingly, the overall individual-level seroprevalence of dairy buffaloes in the study area was 0.72% (9/1,255; 95% CI:0.32-1.36%) while the overall herd-level seroprevalence reached 15.38% (4/26; 95% CI:6.15-33.53%). Moreover, information was gathered on demographic characteristics of the farm owners, individual animals, herd level risk factors and other farm characteristics using a questionnaire. Higher prevalence of *Brucella* spp was observed in buffaloes bred under intensive and semi-intensive feeding systems, compared to those bred under extensive ones. High seroprevalence of brucellosis was also observed in medium size herds. Two of the herds provided available history data of the disease,during the last five years,while, in these two herds, delays in the implementation of brucellosis eradication programs were revealed. Overall, our study indicated that the occurrence of brucellosis in Greece dairy buffalo farms is at a low magnitude. Even though the seroprevalence is low, it can still be a potential hazard for both susceptible animals and humans.

Keywords: brucellosis, buffaloes, seroprevalence, risk factors, Greece

Corresponding Author:
Evanthia J. Petridou, Department of Microbiology and Infectious Diseases
School of VeterinaryMedicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki GR-54124
E-mail address:epetri@vet.auth.gr

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INTRODUCTION

Brucellosis is a zoonosis affecting both public and livestock health worldwide (Pappas et al., 2006). The disease is caused by a facultative intracellular, coccobacillus, non-spore-forming, non-motile bacterium of the genus *Brucella.* Most species of *Brucella* can infect multiple species of animals including humans (Godfroid et al., 2010).

The genus *Brucella* is currently classified into12 known species, according to pathogenicity and host preference basic differences: *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis, B. neotomae, B. pinnipedialis, B. ceti* (Foster et al., 2007;Godfroid et al., 2011; Scholz et al., 2008; Scholz et al., 2010; Scholz et al., 2016), as well asthe more recently proposed *B. microti, B. inopinata, B. papionis* and *B. vulpis* (Whatmore et al., 2014; Scholz et al., 2016).

In humans, brucellosis affects many organs and tissues, while the clinical signs are not specific (Solera et al., 1999; Pappas et al., 2006). Humans are commonly infected either through unpasteurized milk products consumption, or via close contact with infected animals (Mantur et al.,2007). Less often accidental infection may occur due to manipulation procedures of live vaccine strains or virulent *Brucella* strains in the laboratory (Corbel, 1997). Since there is no vaccine available for human brucellosis, prevention relies on control of the disease in animals mainly in species that serve as reservoirs. At the human/animal/ecosystem interface it is critical to reduce opportunities for *Brucella* to spread from one host species to another. This task constitutes a shared responsibility as well as a challenge and the "One Health" approach needs to be implemented (Godfroid et al., 2011).

Regarding cattle, the infection is mainly caused by *B. abortus*, less frequently by *B. melitensis* and occasionally by *B. suis* (OIE, 2016). The pathogenicity of this bacterium is mainly based on its ability to replicate and survive within the host cells. It is considered as biosafety level-3 pathogen while can potentially be exploited as bioweapon (Klietmann et al., 2001).

Bovine brucellosis is manifested with abortions, retained placenta, metritis, weak calves, stillbirth, infertility, and reduced milk yield (Enright, 1990;OIE, 2016). Infected bulls may show signs of infection including orchitis and epididymitis. Chronic orchitis and fibrosis of the testicular parenchyma of infected bulls are frequently followed by impairment of semen production, and partial or permanent infertility (Rhyan et al., 1997;Poester et al., 2013)

All bovines and small ruminants are included in the Greek national brucellosis control and eradication program based on serological tests and slaughtering of the seropositive ones. However, latent infections, prolonged incubation of the pathogen, protection provided by vaccines, and difficulties in distinguishing serologically vaccinated and naturally infected animals have limited the efficacy of the implemented eradication programs. Moreover, the above programs adopt common strategies for both cattle and buffaloes without any distinction among species.

The Rose Bengal Test constitutes the most common serological test for indirect diagnosis of Brucellosis,representing an affordable, quick, simple and efficient methodology for screening herds that can also be used in individual animals as iELISA. On the contrary,the Complement Fixation Test (CFT) is used as a confirmatory test in case of positive agglutination reaction. Although it is complex to perform,it exhibits excellent specificity levels (Godfroid et al., 2010;OIE. 2018).

Regarding the isolation of *Brucella* species and biotypes in humans in Greece, Kansouzidou et al. (1996) and (2002), studied in detail the last 35 years 640 *Brucella* strains of human origin. These studies showed that until the year 2000 *B. melitensis* biotype 2 was the most frequent one, and thereafter, a change occurred and the next years biotype 3 was found to be the primary one. Most human brucellosis cases are attributed to*B. melitensis* (Hadjichristodoulou et al., 1999;Mitka, 2005;Lytras et al., 2016).

In Greece, *B. melitensis* is mostly detected in small ruminants (sheep and goats),whereas *B. abortus* is found in cattle. Different authors have published reports originated from different parts of the country indicating that brucellosis still remains a widespread disease(Sossidou, 1993; Hadjichristodoulou et al., 1999; Minas et al., 2004).

A study has confirmed the presence of *B.abortus*-biovars 1,2,3 and *B.melitensis* biovars1 and 3 examining cultures from milk samples from seropositive bovines and *Brucella melitensis* biovar 3 examining cultures from milk samples from seropositive sheep and goats (Katsiaounis, 1996).

Nevertheless, in Greece, there is lack of information concerning the prevalence the disease in buffalo. Keeping this in mind, the aim of the present study was to investigate the seroprevalence and possible risk factors for brucellosis, in dairy buffaloes in the mainland Greece.

MATERIALS AND METHODS

Study area

The study was carried out from January to August 2018 in northern Greece, where the vast majority of the country's buffalo populations are reared. Blood samples were collected from farms located within Regional Unitsof Thessaloniki, Serres, Kilkis and Rodopi, in Regions of Central Macedonia and Eastern Macedonia and Thrace (Fig.1).

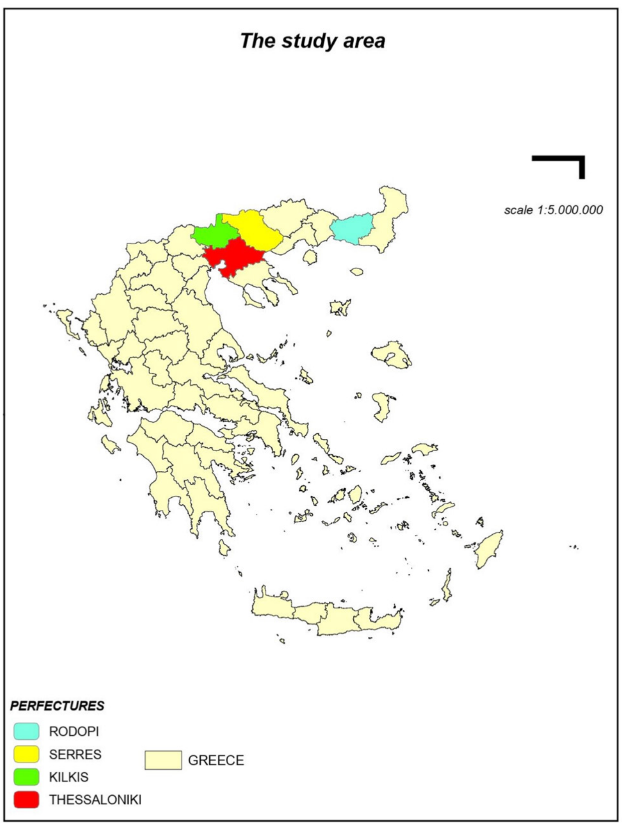

Fig 1: Map of Greece showing the location of the dairy buffaloes' herds

The Region of Central Macedonia is located at 40.6212° N and 23.1918° E while the Region of East Macedonia and Thrace is located at 41.1295° N and 24.8877° E. Both Regions are characterized for the typical Mediterranean climate, having mild and rainy winters, and relatively warm and dry summers while the sunshine duration is long almost all the year.

Study design

The target population of this cross-sectional study was all dairy buffalo herds of the study area. According to the official data obtained from the Greek Ministry of Rural Development and Food, the estimated livestock and herd population in the above Regional Units is recorded at 164,455 bovines and 3,197 bovine herds, respectively and the estimated livestock and herd buffalo population in the above Regional Units is officially recorded at4,006 buffaloes and 34 buffalo herds. Buffaloes aged over than 1-year-old were examined for the needs of this study. Specifically, 26 dairy buffalo herds within selected districts numbered 2,232 buffaloes, while none of the above buffaloes had been vaccinated against brucellosis.

Blood Sampling

A total of 1,255 buffaloes (female and male) > 1 years old, from 26 dairy buffalo herds, were sampled by local official veterinarians according to the procedures of the national brucellosis eradication program. Blood samples were collected aseptically in 4-5 ml plain vacutainer tubes from the jugular or tail vein of each animal.The vacutainer tubes were labeled with the unique herd- animal identification code and were immediately forwarded to the nearest national authorized Veterinary Laboratory. After centrifugation, serum was transferred to cryovials and stored at-20°C until transportation to the Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki for further analyses.

Questionnaire survey

In parallel to blood collection, a structured pilot tested questionnaire designed based on the related literature,was administered by local official veterinarians and the authorized author. The questionnaire was completed within a 10-minute interview of herds' owners, after verbal consent obtained.

The questionnaire was designed to record information concerning potential risk factors for brucellosis infection. It contained questions on demographic characteristic of the owners such as level of education, attendance of relative workshops during the last five years, years of experience in farm, as well as questions on potential herd-level risk factors including herd size, reproductive disorders, housing system, presence of other animal species in the farm, health status of the farm, breeding method, uninterrupted veterinary surveillance of the brucellosis eradication program, management system and biosecurity measures.

Serological Tests

All sera samples collected, were initially screened for the presence of brucella antibodies by Rose Bengal Test (RBT) using the RBT antigen (IDvet,France), according to OIE(2018) procedures**.** Positive and negative control serum for RBT and Complement Fixation Test(CFT) were obtained from Veterinary Center of Thessaloniki. An obvious, clear, and complete agglutination at the end of the 4 min time period was recorded as strong (+++/++++) positive result, whereas clear but not complete agglutination was characterized as medium (++).Absence of agglutination at the end of the 4 min time was marked as negative result. Reactions observed after 4 min were not considered.

Furthermore,CFT was performed to additionally examine all serum samples using standard *B. abortus* Antigen S99(Veterinary Laboratory Agency UK). Complement, haemolysin and antigen were evaluated by titration to determine the working dilutions and the CFT was performed according to the outlined procedures by World Organization for Animal Health(OIE, 2018).

According to the same protocol, sera giving titer (1/4++) equivalent to 20 ICFTU/ml or more were considered to be positive and sera giving titer (1/4+) equivalent to 16.6 ICFTU/ml or less were considered as negative.

Data Analysis

Data obtained from both questionnaire and serology were entered into a Microsoft Excel 2007 file and carefully checked for errors. The statistical analysis was performed using SPSS 2016, version 24 (SPSS Corp., IBM, Armonk., NY, USA). Due to the small sample size of holdings (26) and low prevalence rate of brucellosis on herd's level as well as on animal level, univariate analysis was not performed. However, graph (Fig.2) and descriptive statistics (frequencies) of brucellosis's related risk factors obtained from the questionnaire are presented in Table 1.

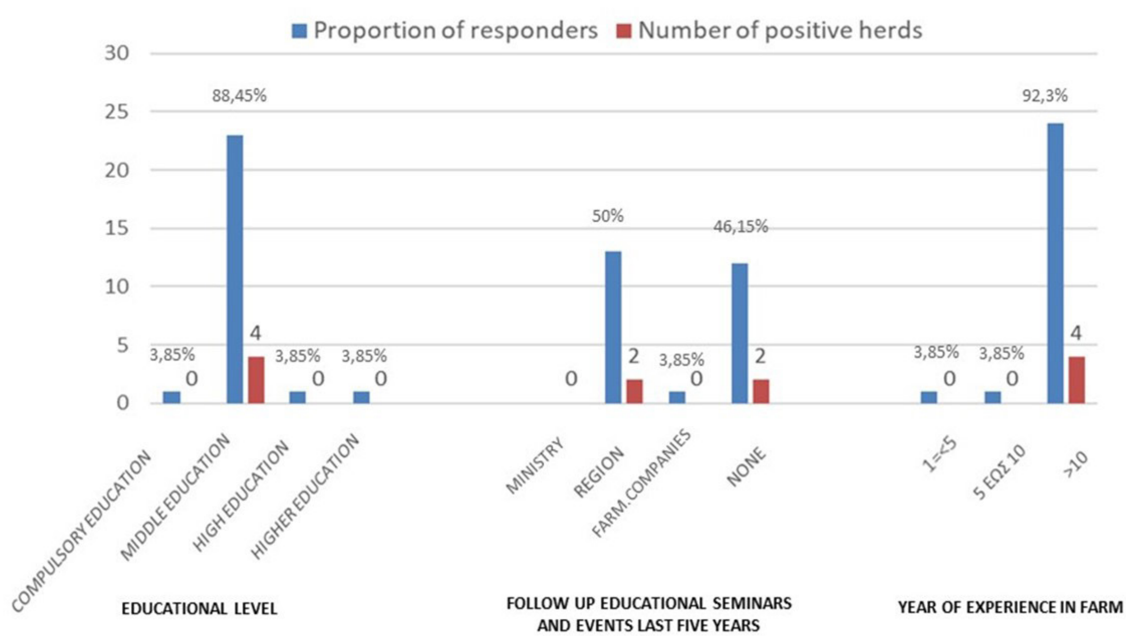

Fig 2: Graphical of educational level, follow up educational seminars and events last five years, year of experience in farm of respondent with positive herds

Table 1: Variables included in the questionnaire addressed to farm owners about buffalo brucellosis

Variables	Response category	Frequency	%
Level of education	1= compulsory	1=1	3.85
	2= middle	2=23	83.85
	3=high	3=1	3.85
	4=higher	4=1	3.85
Attendance of relative workshopsduring the last five yearsorganized by:	1=ministry	1=0	0
	2=region	2=13	50
	3=farms	3= 1	3.85
	4= none	4= 12	46.15
Experience in farm (in years)	1= <5	1=1	3.85
	2= 5 -10	2= 1	3.85
	3=>10	3=24	92.3
Number of buffalos in the farm	1= <50	1=10	38.48
	2=51-100	2=8	30.76
	3=>100	3=8	30.76
Health status of farm	1=B4, B3	1=20	76.9
	2=B2, B+	2=6	23.1
	3=B1, B4 ab*, B3 ab*	3=0	0
Breeding method	1=artificial insemination	1=0	0
	2=natural breeding	2=26	100
	3=both		
Production specialty	1=dairy	1=0	0
	2=meat	2=0	0
	3=combined	3=26	100
Type of operation	1=extensive	1=4	15.4
	2=intensive	2=2	7.7
	3=semi-intensive	3=20	76.9
Waste management	1=composting in tanks	1=0	0
	2=manuredistribution into fields	2=26	100
	3=other	3=0	0
Method of disposing aborted fetuses	1=cremation	1=0	0
	2=burry	2=2	7.7
	3=other (disposalin open field)	3=24	92.3
Method of disposing placentas	1=cremation	1=0	0
	2=burry	2=2	7.7
	3=other (disposalin open field)	3=24	92.3
Dogs in the farm	1=yes	1=17	65.4
	2=no	2=9	34.6
Animals are mainly confined	1=yes	1=10	38.5
	2=no	2=16	61.5
Contact with other sensitive animal species other than bovines (sheep, goats, pigs, dogs, wildlife)	1=yes 2=no	1=17 2=9	65.4 34.6
Distance from nearby infected holdings (in metres)	1=≤500 2= >500	1=16 2=10	61.5 38.5
Workers employed additionally in other farms	1=yes	1=8	30.8
	2=no	2=18	69.2
Use of common pasture	1=yes	1=17	65.4
	2=no	2=9	34.6
Use of common agricultural roads	1=yes	1=16	61.5
	2=no	2=10	38.5
Uninterrupted veterinary surveillance of the eradication program	1=yes 2=no	1=22 2=4	84.6 15.4
Decontamination wheel bath for vehicles	1=yes	1=12	46.2
	2=no	2=14	53.8

Variables	Response category	Frequency	%
Monthly replacement of disinfectants in wheel baths	1=>1, 2=none	1=10 2=16	38.5 61.5
History of disease in the farm	1=yes 2=no	1=2 2=24	7.7 92.3
Type of health problems in animalsduring the lastyear	1=respiratory system 2=digestive system 3=reproductive system 4=other/none	1=0 2=3 3= 4 4= 19	0 11.5 15.4 73.1

*abeyance

RESULTS

Animal and herd-level seroprevalence of Brucellosis in dairy buffaloes in Greece

The total number of buffalo samples that were analyzed using RBT were 1,255.Only 0.72% (9/1,255) were found positive. Among them, 6 RBT-positive samples, that is, 66% (6/9) gave strong positive results and 3 RBT-positive samples, that is, 33.3% (3/9) medium. All the buffalo samples (1,255) were subsequently analyzed with CFT to confirm the presence of antibodies against *Brucella* spp. Among all 1,255 samples, 9 samples were found positive using CFT, that is 0.72% (9/1,255). The overall animal-level seroprevalence was 0.72% (9/1,255, 95% CI: 0.32-1.36%) using both RBT and CFT. The overall herd-level seroprevalence was 15.38%, as 4 out of 26 herds were seropositive (4/26, 95% CI = 6.15-33.53%). A herd was considered as positive if at least one analyzed animal resulted to a positive infection by both RBT and CFT(Table 2 and 3).

Comparison of Serological Tests for Buffalo Brucellosis

The kappa statistics exhibited absolute agreement between RBT and CFT, taking CFT as gold-standard test (Table 4).

Table 2: Animal-level seroprevalence of brucellosis in Buffaloes based on RBT and CFT

Test Assay	Classification	No. Individual	Prevalence %	95% CI
RBT	Negative	1246	0.72% (9/1255)	0.32-1.36%
	Positive	9		
CFT	Negative	1246	0.72% (9/1255)	0.32-1.36%
	Positive	9		
Total		1255	0.72 % (9/1255)	0.32-1.36%

Table 3: Herd-level seroprevalence of Buffaloes brucellosis based on RBT and CFT

Test assay	Classification	No. herds	Prevalence %	**95% CI**
RBT	Negative	22	15.3% (4/26)	6.15-33.53%
	Positive	4		
CFT	Negative	22	15.3% (4/26)	6.15-33.53%
	Positive	4		
Total		26	15.3% (4/26)	6.15-33.53%

Table 4: Kappa test for agreement between RBT and CFT for Buffalo brucellosis

		CFT +ve -ve	Kappa value	Kappa interpretation	p-value
RBT	Positive	9 0		excellent agreement	<0.00
	Negative	0 1246	1		
TOTAL		9 1246			

Interpretation of kappa statistic: > 0.8-1: excellent agreement; > 0.6-0.8: substantial agreement; >0.4-0.6: moderate agreement; >0.2-0.4: fair agreement; > 0-0.2: slight agreement; 0: poor agreement; <0: disagreement

Results of Questionnaire Survey

Among the 26 interviewed farmers investigated during our survey, the majority (88.45%)declared to have middle educational level, and more than 10 years of professional experience in farms(92.3%)(Fig.2).

Seventy-six-point nine percent (76,9%) of the holdings belonged to official brucellosis free status,while the remaining 23.1% belonged to *Brucella* positive ones (Table1).Of the total respondents, 24livestock farmers (92.3%)declared that the last 5 years no brucellosis history in the farm has been observed and all animals of the herd were free of pathogens.Sixty-five percent (65%) of the farmers owed dogs within the farms while due to shared common roads and pastures all animals of the flock could potentially comein close contact with other wild animals from the forest. Also, 61% answered that they never change the disinfectants in the decontamination wheel bath for vehicles. Moreover, it was revealed that the waste management method used was manure disposal by discharging onto pasture as illustrated in Table 1.

DISCUSSION

Buffaloes constitute a traditional component of the livestock heritage of Greece and are considered as important part of the national domestic livestock genetic resources and biodiversity. Although buffaloes can act as an important reservoir of brucellosis for the bovine species, there is a lack of information on the regional prevalence and distribution of the disease in buffaloes in Greece.

The present study revealed that the overall individual seroprevalence of dairy buffalo brucellosis in Greece was at low levels, i.e. 0.72% (9/1,255, 95% CI:0.32-1.36%) and the herd level seroprevalence was at 15.3% (4/26, 95% CI:6.15-33.53%). Epidemiological studies have recommended the use of two tests applied serially to maximize results accuracy (Godfroid et al., 2002). A combination of RBT and CFTis the most widely accepted serial testing scheme. RBT is highly sensitive test and could easily be applied in field conditions, whereas CFT is highly specific, usually utilized as a confirmatory test method (Mainar-Jaime et al. 2005; Samui et al., 2007; OIE, 2018).Nine positive serum samples in the present study were found strongly positive in both RBT and CFT tests.

Seroprevalence (0.72%) was below the 1% seroprevalence of brucellosis reported using the RBT and CFT in all dairy bovine in Regional Units of Thessaloniki, Kilkis, Serres and Rodopi according to the official data from Greek Ministry of Rural Development and Food, 2018. A previous experimental study of intra-conjunctival inoculation of *B. abortus* 1969D strain suggested that buffaloes are more resistant to *B. abortus* infection than cattle (Adesiyun et al., 2010). Nevertheless, a study conducted in Trinidad and Tobago showed that buffaloes tend to be infected with *Brucella abortus* strain less virulent than cattle. Therefore, in addition to apparent innate resistance to infection, buffaloes tend to be infected with less virulent strains (Adesiyun et al., 2011). Yantzis (1984) reported 4.7% to 1.5% seroprevalence of cattlelevel brucellosis from Central Macedonia in Greece for the years 1977-1981.

Regarding the species and biotypes of brucellosis affecting bovine animals in Greece, the data indicate that since 1926, when the first outbreak of bovine brucellosis was diagnosed, *B. abortus* biovars 1, 2 and 3 and *B. melitensis* biovars 1 and 3 strains were isolated by culture of milk specimens(Katsiaounis, 1996).

The herd level seroprevalence of 15.3% was higher than the 3.3% reported from the same Regional Units according to the official data obtained from the Greek Ministry of Rural Development and Food for the year 2018,implying that the *Brucella* infection was probably extended this period possibly on account of non-effective biosecurity measures. Differences in the herd level seroprevalence rates observed in this study, as opposed to those recorded by official data, may be owing to several factors such as the presence or absence of infectious foci, including *Brucella*-infected dairy farms or beef cattle farms in the surrounding areas.

Various reports have been published from different countries.Seroprevalence of cattle brucellosis was reported to be 6.5% in Jordan (Al-Majali et al. 2009). In Punjab India, the prevalence of disease in buffalo and cattle increased to 16.4 and 20.7%, respectively, with an overall prevalence of brucellosis at 18.3% (Aulakh et al. 2008). In Ethiopia, results showed that the overall seroprevalence of bovine brucellosis at the individual animal level was 2.9% (low) and the overall seroprevalence at the herd level was 13.6% (moderate) (Jergefa et al., 2009). In Albania, the overall herd prevalence of bovine brucellosis in beef cattle, based on the results of RBT, FPA, and ELISA, was 55% (CI>0.95, 40-71%) (Fero et al., 2020).

Recently, in North Brazil, the area with the largest buffalo herd in the country, and more specifically the state of Pará, (Silva et al., 2014b) evaluated 3,917 serum samples from pregnant and non-pregnant buffalo cows using RBT, found that 4.8% (188/3,917) of the animals were seropositive. Of these, 95.7% (180/188) were confirmed by 2-Mercapteoethanol test (2-ME), showing that the infection is active in the Brazilian region with the largest buffalo population and the disease poses a risk to public health and buffalo production in the Amazon biome. In 2013, in north-east Argentina, Konrad et al., (2013) reported 6.4% positive buffaloes by FPA. Despite the implementation of eradication measures, brucellosis in buffaloes in Italy, especially in the province of Caserta, remains at high prevalence (Caporale et al., 2010).

In 2005, Ligda and Georgoudis, concluded that the buffalo farmers rear their buffaloes following traditional ways and reported that the buffalo rearing system in Greece was grazing during the entire year, whereas during the period from November to April, supplementary feed was administered to the animals.

In this study, high prevalence of *Brucella* was observed in buffaloes which were reared under intensive and semi-intensive production systems, in contrast to those bred under extensive ones. This finding is in accordance with the study of Tsegayea et al., in Ethiopia in 2016. This could be explained by the fact that in these feeding systems there is a greater chance of contact among infected and healthy animals, or healthy animals with infectious materials, since most farmers do not follow good practices in terms of biosecurity and hygiene (Tsegayea et al., 2016).

In our study a high number of positive herds was observed in medium herds (51-200 animals) as illustrated in Table 5. The association between the presence of bovine brucellosis and the herd size, corroborated the results of studies in the exciting literature (Al-Majali et al., 2009;De AlencarMota et al., 2016). It was also observed that approximately 65% of the herds shared common pasture, common road and they were less than 500 meters away from nearby already infected holdings. Cleaning and disinfection procedures of premises and manure were not consistently applied leading to posing high risks of transmitting the disease within and in between the herds.This finding is in accordance to other studies in extensive livestock production system in Ethiopia(Megersa et al., 2011).

Also, 61% of the herds were not"closed" and therefore the dynamics and frequent migration of pastoral herds might increase the risk of coming into close contact with other potentially infected herds as well as being exposed to geographically limited or other diseases. Animal movements also increase the chance of contact with wild animals (Valergakis et al.,2008). Muma et al. (2007),demonstrated that herds coming into close contact with wildlife had higher probability to be infected than those without contact. The presence of dogs has been described as a potential risk factor for brucellosis infection in farm animals, especially in endemic areas and/or areas where there are no brucellosis control programs. Dogs act as mechanical carriers that feed on aborted fetus and placentas. Dogs acting as mechanical carriers feeding on aborted fetus and placentas,spread bacteria into the environment (Coelho et al., 2015).

Two of the herds had history in brucellosis during the last five years and in these two herds there were delays in the implementation of brucellosis eradication programs. Due to the small sample of farms in the study area, inter-farm transmission factors and farm-level variables (common management practices, such as disposing aborted fetuses and placentas, rearing other animals within the farm etc.), were not investigated by statistical models. Thus, all buffalo herds and all buffaloes older than one year in each herd were included without random sampling. Only serological studies were conducted, and it was not supported with the gold standard test of bacterial isolation and identification.

Table 5: Number of positive herds based on RBT and CFT, according to herd size.

Prefectures	Number of herds	Herd size (%)			Number of positive herds		
		Small (1-50)	Medium (51-200)	Large (>201)	Small (1-50)	Medium (51-200)	Large (>201)
Thessaloniki	5	2	3	0	0	2	0
Serres	16	6	8	2	0	1	2
Kilkis	3	1	2	0	0	0	0
Rodopi	2	1	1	0	0	0	0
TOTAL	26	10(38.4)	14(53.8)	2(7.8)	0	3	1

CONCLUSION

In conclusion, the present study indicated that the occurrence of brucellosis in Greece dairy buffalo farms is at a low magnitude. Although the seroprevalence is low, it can still be a potential hazard for both animals and humans.Moreover, the existence of distinct epidemiological characteristics between buffalo and bovine brucellosis must be taken into consideration while further studies on this topic are needed, since most of the published papers are referred to the bovine species.

ETHICAL APPROVAL: All animal manipulations were carried out according to the EU Directive on the protection of animals' usage for scientific purposes (2010/63/EU). The research protocol was approved by the General Assembly of the Veterinary Faculty of Aristotle University of Thessaloniki decision 48/9-12-2014

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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