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Isolation and characterisation of *Brucella melitensis* by bacteriological and molecular methods from livestock in North Cyprus

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Abstract: In this study, the isolation, biotyping and molecular characterisation of *Brucella melitensis* from cattle, sheep and goats in North Cyprus are reported on. A total of 319 raw milk samples obtained from seropositive dairy livestock (190 cattle, 74 sheep and 55 goats) and tissue samples including the liver, spleen and abomasal contents obtained from 32 aborted foetal samples (5 cattle, 18 sheep and 9 goats) were analysed for the presence and characterisation of the agent. *B. melitensis* was isolated and identified from 90 out of 319 (28.2%) milk and 19 out of 32 (59.4%) foetal samples by conventional bacteriological methods. Identification of all 109 isolates was confirmed by using real-time PCR with genus and species-specific primers. Following the preliminary identification, 27 selected isolates representing various counties and herds were further analysed by conventional methods. Twenty (74.1%) isolates were identified as *B. melitensis* biovar 1 and seven (25.9%) were identified as *B. melitensis* biovar 3. The Bruce-ladder multiplex PCR assay revealed that all the isolates were field strains. The results of the present study confirmed the presence of *B. melitensis* in livestock including the cattle population in North Cyprus. Even though the majority of the samples came from seropositive cattle, *Brucella abortus* was not isolated in the study. The results also revealed the potential public health risk of brucellosis in livestock emphasising the need of implementing strict control and eradication strategies against the disease in animal populations in order to protect human health.

Keywords: aborted foetuses; biotyping; Bruce-ladder multiplex PCR; raw milk; real-time PCR

Brucellosis is an important notifiable bacterial zoonotic disease affecting various animal species, mainly livestock, and humans worldwide (Cvetnic et al. 2015; Caine et al. 2017; De Massis et al. 2019). The disease is caused by members of the genus of *Brucella*. Currently, there are 12 closely related species that are recognised within the genus by means of both genetic and immunological traits.

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Nevertheless, each one of these species shows individual host preferences, pathogenicity, and epidemiology, and they are divided into two groups, namely, six classical *Brucella* nomenspecies, and the more recently defined species within the last two decades. Among the six classical *Brucella* nomenspecies, *Brucella abortus* (with 7 biovars) mainly infects cattle; *B. melitensis* (with 3 biovars) affects sheep and goats; *B. suis* (with 5 biovars) infects pigs; *B. canis* affects dogs; *B. ovis* particularly affects rams; and *B. neotomae* infects the desert wood rat. On the other hand, the more recently defined *Brucella* species include *B. microti* isolated from the common vole (*Microtus arvalis*); *B. ceti* isolated from marine mammals; *B. pinnipedialis* isolated from seals; *B. inopinata* isolated from a human breast implant infection; *B. papionis* isolated from baboons (*Papio* spp.); and *B. vulpis* isolated from the red fox (*Vulpes vulpes*) (OIE 2018; De Massis et al. 2019; Fero et al. 2020).

Brucellosis in livestock, principally caused by *B. abortus* and *B. melitensis* in cattle and small ruminants, respectively, poses a risk of huge economic losses as a result of abortion, infertility, and other related reproductive disorders as well as a reduced milk yield (Caine et al. 2017; Khan and Zahoor 2018; Shalaby et al. 2019). Apart from leading to economic impacts, these animals are also considered to be the most common source of human brucellosis, a major public health concern resulting with the acquisition of agents via unpasteurised milk, meat and by-products of infected animals (Cvetnic et al. 2015; Caine et al. 2017; Khan and Zahoor 2018).

Brucellosis, caused by *B. abortus* and/or *B. melitensis*, shows a widespread distribution globally in livestock, except in certain areas where the disease has been eradicated such as some countries in northern and central Europe, as well as in Canada, Japan, Australia and New Zealand (OIE 2018). Both bovine brucellosis caused mainly by *B. abortus* and ovine and caprine brucellosis caused by *B. melitensis* tend to be endemic, and are considered as one of the most important zoonotic infections in the Mediterranean basin (Khan and Zahoor 2018; OIE 2018; De Massis et al. 2019).

According to official records, the very first cases of brucellosis, due to *B. abortus* and *B. melitensis* were introduced to the isle of Cyprus in 1921 and the early 1930s via dairy cattle imported from England and dairy goats imported from Malta, respectively. Following eradication of both diseases in 1932, another *B. melitensis* outbreak occurred in the domestic goat population due to imported Damascus goats from Syria in 1960. During this second outbreak, *B. melitensis* biovar 1 was also defined in cattle herds, possibly due to cross-contamination, and it was adopted that *B. melitensis* was also the main causative agent of bovine brucellosis in Cyprus (Republic of Cyprus 1980). Since then, brucellosis in livestock has tended to be endemic in Northern Cyprus. Following a stagnant period, implementation of a project named "Technical assistance to strengthen the capacity of Turkish Cypriot Veterinary Services to eradicate, control and prevent animal diseases (Europe Aid/138353/DH/SER/CY)" in 2015, accelerated the National Brucellosis Monitoring and Control Programmes for livestock including isolation and characterisation of the possible aetiological agents circulating in the herds. The results of the second cycle sampling, covering 39 377 cattle and 259 503 sheep and goats by the end of 2019 revealed a herd and individual seroprevalence of 8.29% and 1.34% for bovine, and, 6.17% and 1.17% for ovine and caprine brucellosis, respectively (data unpublished).

Despite the fact that brucellosis in livestock is endemic in the Turkish Republic of Northern Cyprus (TRNC), there is a lack of scientific data published on this subject. The only data concerning brucellosis were published by Ozdogac et al. (2018), in which a seropositivity ranging from 3.1% to 6.5% was recorded among humans including veterinarians, animal keepers and butchers. These figures comply with the aforementioned low to moderate seroprevalence findings obtained from livestock, regardless of the causative agent.

Therefore, the aim of the study reported herein was to determine the possible causative agents of brucellosis in livestock and to identify the species and biovars by using bacteriological and molecular techniques in Northern Cyprus.

MATERIAL AND METHODS

Sample collection

A total of 32 aborted foetuses (5 from cattle, 18 from sheep, and 9 from goats) and 319 milk samples (190 from cattle, 74 from sheep, and 55 from goats) were used in this study for the iso-

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lation of field strains of *Brucella* sp. The aborted foetuses were submitted by the farmers to the Directorate of Veterinary Department, the Ministry of Agriculture and Natural Resources in Nicosia, the Turkish Republic of Northern Cyprus, for routine bacteriological diagnosis. Upon arrival on ice, all the foetuses were subjected to a necropsy and tissue samples including the liver, spleen and abomasal contents were collected aseptically and analysed on the same day. Milk samples were collected under aseptic conditions from dairy animals that were found to be serologically positive within the scope of the "Brucellosis Surveillance, Control, and Eradication Campaign" carried out by the same Directorate. About 10 ml of the mid-stream milk samples were collected into sterile screw capped tubes and transferred to the laboratory under appropriate conditions and analysed on the same day.

Details of the samples analysed concerning the individual and herd distribution are summarised in Table 1.

Growth media and culture conditions

In order to increase the sensitivity of the diagnosis by cultural isolation, both Farrell's medium (FM) and CITA medium were used simultaneously.

Farrell's medium was prepared using a *Brucella* Medium Base (Oxoid, CM0169) supplemented with a *Brucella* Selective Supplement (Oxoid, SR0083A) containing Polymixin B 2 500 IU, bacitracin 12 500 IU, cycloheximide 50.0 mg, nalidixic acid 2.5 mg, nystatin 50 000 IU and vancomycin 10.0 mg per 500 ml medium, together with a 5% sterile inactivated horse serum (Oxoid, SR0035C), and 2% of a filter sterilised solution of dextrose. The liquid FM, which is used for the enrichment of the milk samples, was prepared in the same way as described above, except replacing *Brucella* medium base with Triptone Soy Broth (Oxoid, CM0129).

Table 1. Number of samples analysed in the study

Species	Foetal samples			Milk samples		
	herd	individual	sample	herd	individual	sample
Cattle	5	5	5	11	104	190
Sheep	10	15	18	7	47	74
Goat	5	7	9	5	29	55
Total	20	27	32	23	180	319

The CITA medium was prepared using a Blood Agar Base (Oxoid CM0055) supplemented with an antibiotic supplement comprised of 20 mg of vancomycin (Sigma V-2002), 7.5 mg of colistin (Sigma C-1511), 17.7 mg of nystatine (Sigma N-3503), 10 mg of nitrofurantoin (Sigma N-7878), 4 mg of amphotericin B (Sigma A-4888), 5 ml of methanol (Merck 1719107 348), 1 ml of dimethyl sulfoxide (Merck K43987812 308) per litre of medium, together with a 5% sterile inactivated horse serum (Oxoid SR0035), and a 2% filter sterilised dextrose solution. The liquid CITA medium, used for the enrichment of the milk samples, was prepared in the same way as the procedure described above, except replacing blood agar base with Triptone Soy Broth (Oxoid, CM0129), and using a 10% horse serum.

All the media were prepared according to the directions of the manufacturers and sterilised by autoclaving at 121 °C for 15 minutes. After sterilisation, all the supplements were added under aseptic conditions after the media were cooled to approximately 56 °C. All the bacteriological analyses were performed in Biological Safety Level-3 (BSL-3) Bacteriology Laboratory of the Directorate of Veterinary Department, as has been advised elsewhere (OIE 2009). Following centrifugation at 3 824 *g* for 15 min, the cream and deposit of the milk samples and aseptically obtained foetal tissue samples were inoculated on Farrell's and CITA media simultaneously. All the inoculated plates were incubated at 37 °C both with and without the presence of 5–10% CO_2 for at least 2 weeks. A Thermo Scientific Heracell 150i CO_2 incubator (Langenselbold, Germany) was used for the incubation.

Identification and biotyping of the isolates

The preliminary identification of the *Brucella* isolates was carried out by examining the following tests; macroscopic and microscopic morphology, urease and oxidase tests, and slide agglutination test with a polyclonal anti-*Brucella* serum, as described previously (OIE 2018; Saavedra et al. 2019; Shalaby et al. 2019). The isolates with typical smooth colony morphology showing Gram-negative coccobacilli in the Gram-stained smears and positive for urease-, oxidase- and slide agglutination test were identified as *Brucella* sp., and they were stored at –18 °C in a Tryptone Soy Broth

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(Oxoid CM0129) supplemented with 10% glycerine for further characterisation.

The species and biovar identification of the presumptive isolates were carried out in the *Brucella* Reference Diagnostic and Vaccine Production Laboratory of İstanbul Pendik Veterinary Control Institute, Republic of Turkey Ministry of Agriculture and Forestry. The characteristics, such as the CO_2 requirement for growth, production of H_2S, growth in the presence of basic fuchsin and thionin, Tbilisi and R/C phage lysis, growth in the presence of safranin O and agglutination with A and M monospecific sera, were analysed as described previously (OIE 2018; Saavedra et al. 2019; Shalaby et al. 2019).

Molecular characterisation

DNA EXTRACTION

The DNA from the *Brucella* isolates were extracted with the method described by The World Organisation for Animal Health (OIE 2009). Briefly, pure bacterial cultures (one colony from each isolate) were transferred to 200 µl of sterile physiological saline with an inoculating loop, boiled for 10 min in a water bath and centrifuged at 12 000 *g* for 20 seconds. Then 1 µl of supernatant from each suspension was used as the DNA template, corresponding approximately to 0.1–0.05 µg/µl DNA, in the polymerase chain reaction (PCR) amplifications. During the DNA extraction studies, both laboratory-defined *Escherichia coli* strains and sterile distilled water were used as the negative controls in order to check the cross-contaminations.

REAL-TIME PCR

A real-time PCR (RT-PCR) assay was performed to confirm the presence of the genus *Brucella* and to identify *B. melitensis* in the DNA samples extracted from the cultures grown. The RT-PCR assay was carried out using a Qiagen OneStep RT-PCR commercial kit (Cat. No. 210212), Biotium Eva Green Dye (Cat. No. 3100) and a Qiagen Rotor-Gene Q Real-Time PCR device (Hilden, Germany). The DNA lysates prepared from standard *B. abortus* (MVMAU101) and *B. melitensis* (MVMAU102) strains obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University were used as the positive controls; whereas, the DNA lysates prepared from an in-house isolated *Escherichia coli* strain was used as the negative control. Similarly, the *Brucella* genus and *B. melitensis* species specific primers (Sareyyupoglu et al. 2008) that were used in the RT-PCR were kindly supplied by the same department (Table 2). The RT-PCR assay was carried out in 20 µl of a reaction mixture containing 10 µl of the 2 X reaction mix, 6 µl of the PCR water, 1 µl of Eva green, 0.5 µl of a forward primer (10 µM), 0.5 µl of a reverse primer (10 µM), and 2 µl of the DNA template. The thermocycling was run with the following conditions: 1 cycle of hot start enzyme activation at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing at 55 °C for 20 s, with data acquired at the polymerisation step at 72 °C for 15 s in the green channel. After amplification, a high-resolution melt was performed between 60 and 90 °C at a rate of 0.02 °C per step.

BRUCE-LADDER MULTIPLEX PCR

The Bruce-ladder multiplex PCR assay was performed by using eight pairs of species-specific oligonucleotide primers as has been previously reported (Sumathi et al. 2019; Tekle et al. 2019). Briefly, the Bruce-ladder multiplex PCR reaction was conducted with a final volume of 25 µl of the PCR reaction mix containing 2.5 µl of 10 × PCR buffer, 5.0 µl of dNTPs (2 mM) 400 µM each one, 1.5 µl of Mg^{2+}

Table 2. *Brucella* genus (Bru3F/Bru3R) and *B. melitensis* species (BrumelF/BrumelR) specific primers that were used in the RT-PCR (Sareyyupoglu et al. 2008)

Primer name	Sequence (5'→3')	Primer size (bp)	Amplicon size (bp)	Target gene
Bru3F	TAACCCCGGATATGAATCTGAAC	23	170	*IS711*
Bru3R	TGGATATTACTGCTCTACCTTCTGTG	26	170	*IS711*
BrumelF	TAGCTTACCCGCCAATCTTC	20	245	*IS711*
BrumelR	GCGCTATGATCTGGTTACGTTG	22	245	*IS711*

RT-PCR = real-time polymerase chain reaction

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(50 mM) 3.0 mM, 7.6 µl of a Bruce-ladder eight pair primer cocktail (12.5 µM) 6.25 pmol of each one, 0.3 µl of DNA polymerase 1.5 IU, 7.1 µl of H_2O (PCR-grade) and 1 µl of genomic DNA from the sample. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 7 min, followed by 25 cycles of template denaturation at 95 °C for 35 s, primer annealing at 64 °C for 45 s and primer extension at 72 °C for 3 min, followed by a final extension at 72 °C for 6 minutes. The amplified products were visualised under ultraviolet (UV) light by electrophoresis using a 1.5% agarose gel run at 130 V for 50 min followed by staining with ethidium bromide.

RESULTS

Isolation, identification and biotyping of *B. melitensis*

As shown in Table 1, a total of 32 foetal samples and 319 milk samples obtained from the livestock were plated onto the FM and CITA media for the isolation. Following incubation at 37 °C with and without CO_2, colonies appeared to be visible from 48 h and onwards. Colonies were observed only in the plates incubated without the presence of CO_2. The isolates displaying a typical macroscopic and microscopic morphology with positive oxidase-, urease- and slide agglutination test results with polyclonal anti-*Brucella* serum were defined as *Brucella* sp. A total of 19 foetal and milk samples yielded *Brucella* isolates. The isolates were subcultured to fresh media for further characterisation and molecular differentiation. Details of the preliminary isolation results of the foetal and milk samples are shown in Tables 3 and 4.

Following the preliminary identification, 27 selected isolates representing various counties and herds were further analysed with conventional methods and biotyped as 20 (74.1%) *B. melitensis* biovar 1 and seven (25.9%) *B. melitensis* biovar 3. The results of the biotyping are outlined in Table 5. No other *Brucella* sp. and/or biovars were detected.

Phenotypic characteristics that were considered for biotyping of 27 *Brucella melitensis* isolates are summarised in Table 6.

Real-time PCR

In the *Brucella* genus specific RT-PCR studies, the cycle threshold (ct) value of all the samples was

Table 3. Isolation results of *Brucella* sp. from the aborted foetuses

Species	Foetus			Isolation (%)		
	herd	individual	sample	herd	individual	sample
Cattle	5	5	5	4 (80.0)	4 (80.0)	4 (80.0)
Sheep	10	15	18	5 (50.0)	7 (46.7)	12 (66.7)
Goat	5	7	9	2 (40.0)	2 (28.6)	3 (33.3)
Total	20	27	32	11 (55.0)	13 (48.1)	19 (59.4)

Table 4. Isolation results of *Brucella* sp. from the milk samples

Species	Milk			Isolation (%)		
	herd	individual	sample	herd	individual	sample
Cattle	11	104	190	9 (81.8)	39 (37.5)	8 (35.8)
Sheep	7	47	74	7 (100.0)	17 (36.2)	17 (23.0)
Goat	5	29	55	5 (100.0)	5 (17.2)	5 (9.1)
Total	23	180	319	21 (91.3)	61 (33.9)	90 (28.2)

Table 5. Results of biotyping the *B. melitensis* isolates

Species	Isolates	*B. melitensis* biovar 1 (%)	*B. melitensis* biovar 3 (%)
Cattle	13	8 (61.5)	5 (38.5)
Sheep	10	9 (90)	1 (10)
Goat	4	3 (75.0)	1 (25)
Total	27	20 (74.1)	7 (25.9)

Table 6. Phenotypic characteristics of the *Brucella melitensis* isolates (adopted from Quinn et al. 1994)

No. of isolates	CO_2	H_2S	Urease	Growth in the presence of dyes		Lysis by Tbilisi phage		Monospecific sera			Conclusion
				thionin 20 µg/ml	fuchsin 20 µg/ml	RTD	10^4 × RTD	A	M	R	
20	–	–	> 20 h	+*	+	–	–	+	–	–	biovar 1
7	–	–	> 20 h	+*	+	–	–	+	+	–	biovar 3

*Most isolates are positive; (+) = positive; (–) = negative
RTD = routine test dilution

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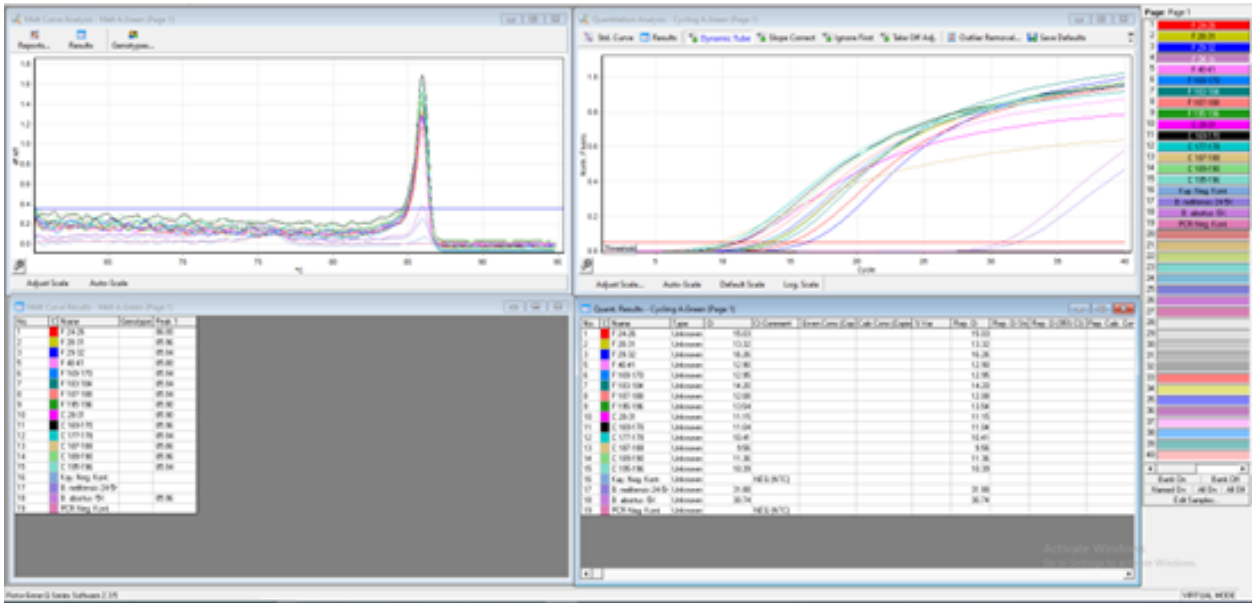

Figure 1. Screenshot of the optimisation studies of the *Brucella* genus specific RT-PCR with a OneStep RT-PCR kit
RT-PCR = real-time polymerase chain reaction

observed as a value between 9.56 and 16.26. *B. melitensis* –5X dilution lysate (ct 31.88) and *B. abortus* –5X dilution lysate (ct 30.74) used as the positive controls were evaluated as positive. The *E. coli* strain yielded a negative result. All of the samples and positive controls showed a melt curve at around 85.90 °C. As a result of the RT-PCR study, all the samples were confirmed to belong to the *Brucella* genus (Figure 1). In the *Brucella melitensis* species specific RT-PCR studies, the ct value of all the samples was observed as a value between 9.67 and 15.71. *B. melitensis* –4X dilution lysate (ct 27.14) used as the positive control was determined as positive. The *B. abortus* lysate and *E. coli* strain were detected as negative. All of the samples and all of the positive controls showed a melt curve at around 87.10 °C. As a result of the RT-PCR study, all the samples were confirmed to be *B. melitensis* (Figure 2).

All the investigated lysates were positive in the RT-PCR assays using both the *Brucella* genus spe-

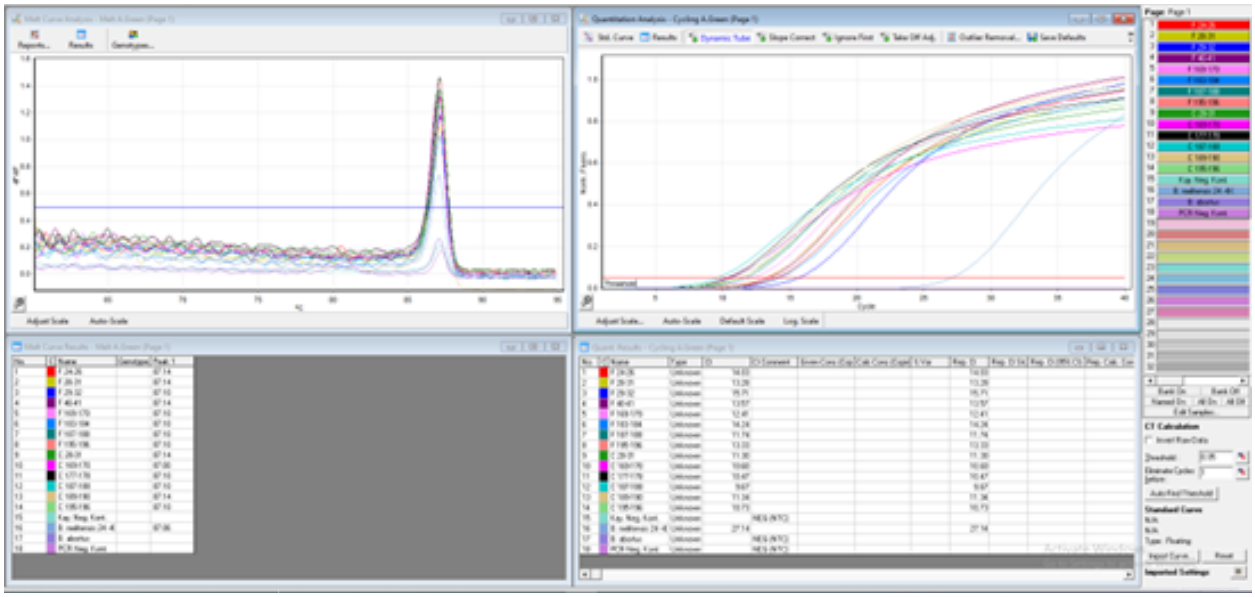

Figure 2. Screenshot of the optimisation studies of the *B. melitensis* species specific RT-PCR with a OneStep RT-PCR kit
RT-PCR = real-time polymerase chain reaction

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Table 7. RT-PCR results of the *Brucella* isolates

Species	Foetal culture positive samples			Milk culture positive samples			Identification result
	herd	individual	sample	herd	individual	sample	*B. melitensis*
Cattle	4	4	4	9	39	68	*B. melitensis*
Sheep	5	7	12	7	17	17	*B. melitensis*
Goat	2	2	3	5	5	5	*B. melitensis*
Total	11	13	19	21	61	90	*B. melitensis*

RT-PCR = real-time polymerase chain reaction

cific and *B. melitensis* species specific primers. Therefore, all 109 isolates (72 cattle, 29 sheep, 8 goat) analysed in this study were confirmed as *B. melitensis*. Detailed results of the isolates on the herd, individual and sample basis are given in Table 7.

Bruce-ladder multiplex PCR assay

All 27 of the selected isolates biotyped were also concurrently subjected to genotypic characterisation and differentiation, and all of them were confirmed as *B. melitensis* field strains by the Bruce-ladder multiplex PCR assay. All the isolates, including 4 bovine, 10 ovine, and 13 caprine strains, yielded amplicons of 1 682 bp, 1 071 bp, 794 bp, 587 bp, 450 bp and 152 bp band size fragments. The amplification profiles of the field isolates of *B. melitensis* and positive control of *B. melitensis* Ether reference strain (ATCC 23458) are illustrated in Figure 3. Although the amplification of the 1 682 bp fragment was observed weakly in one isolate (Figure 3, Lane 19), it was still regarded as *B. melitensis* due to its microbiological and biochemical characteristics. The primers that were used in the Bruce-ladder multiplex PCR assay are shown in Table 8.

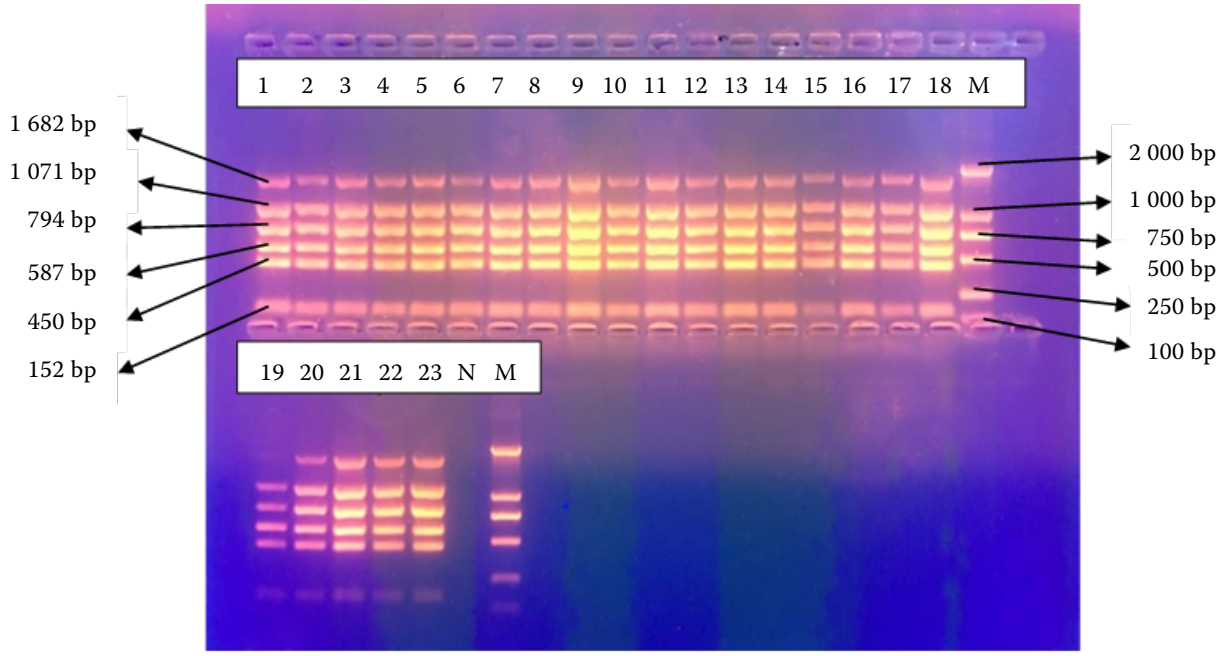

Figure 3. The amplification profiles of the field strains of *B. melitensis* and reference strain in the Bruce-ladder multiplex PCR assay

Lane 1: positive control (*B. melitensis* Ether reference strain, ATCC 23458); Lanes 2–23: field isolates; Lane M: marker (2 000 bp); Lane N: negative control

PCR = polymerase chain reaction

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Table 8. Oligonucleotides used in the Bruce-ladder multiplex PCR assay together with the amplicon sizes, DNA targets and sources of genetic differences (Sumathi et al. 2019)

Primer	Sequence (5'→3')	Amplicon size (bp)	DNA targets	Source of genetic differences
BMEI0998f BMEI0997r	ATC-CTA-TTG-CCC-CGA-TAA-GG GCT-TCG-CAT-TTT-CAC-TGT-AGC	1 682	glycosyltransferase, gene *wboA*	*IS711* insertion in BMEI0998 in *B. abortus* RB51, and deletion of 15 079 bp in BMEI0993–BMEI1012 in *B. ovis*
BMEI0535f BMEI0536r	GCG-CAT-TCT-TCG-GTT-ATG-AA CGC-AGG-CGA-AAA-CAG-CTA-TAA	450	immunodominant antigen, gene *bp26*	*IS711* insertion in BMEI0535–BMEI0536 in *Brucella* strains isolated from marine mammals
BMEII0843f BMEII0844r	TTT-ACA-CAG-GCA-ATC-CAG-CA GCG-TCC-AGT-TGT-TGT-TGA-TG	1 071	outer membrane protein, gene *omp31*	deletion of 25 061 bp in BMEII826–BMEII0850 in *B. abortus*
BMEI1436f BMEI1435r	ACG-CAG-ACG-ACC-TTC-GGT-AT TTT-ATC-CAT-CGC-CCT-GTC-AC	794	polysaccharide deacetylase	deletion of 976 bp in BMEI1435 in *B. canis*
BMEII0428f BMEII0428r	GCC-GCT-ATT-ATG-TGG-ACT-GG AAT-GAC-TTC-ACG-GTC-GTT-CG	587	erythritol catabolism, gene *eryC* (derythrulose-1-phosphate ehydrogenase)	deletion of 702 bp in BMEII0427–BMEII0428 in *B. abortus* S19
BR0953f BR0953r	GGA-ACA-CTA-CGC-CAC-CTT-GT GAT-GGA-GCA-AAC-GCT-GAA-G	272	ABC transporter binding protein	deletion of 2 653 bp in BR0951–BR0955 in *B. melitensis* and *B. abortus*
BMEI0752f BMEI0752r	CAG-GCA-AAC-CCT-CAG-AAG-C GAT-GTG-GTA-ACG-CAC-ACC-AA	218	ribosomal protein S12, gene *rpsL*	point mutation in BMEI0752 in *B. melitensis* Rev 1
BMEII0987f BMEII0987r	CGC-AGA-CAG-TGA-CCA-TCA-AA GTA-TTC-AGC-CCC-CGT-TAC-CT	152	transcriptional regulator, CRP family	deletion of 2 203 bp in BMEII0986–BMEII0988 in *B. neotomae*

PCR = polymerase chain reaction

DISCUSSION

Livestock breading is one of the main economic sources of income in Northern Cyprus, where the main farm animals are sheep, followed by goats and cattle with an estimated population of 193 635, 84 131, and 68 458, respectively. Brucellosis is a notifiable disease and its presence and endemic course have been well documented by serosurveys performed on a routine basis by the Directorate of Veterinary Department. Until recently, almost all studies carried out on animal brucellosis were based solely on serological tests without the bacteriological identification of the causative agent. In Northern Cyprus, farm animals are tested for brucellosis as part of a national eradication programme and culled if they test positive. A vaccination campaign is not practiced; instead, a test and slaughter measure is applied. In this respect, although the disease has been controlled to a certain extent throughout the country, due to some financial constraints, the overall eradication has not been accomplished yet.

Humans can get the infection via ingestion of unpasteurised milk or milk products, and through contact with infected cattle or their discharges. Interaction of the mucosa and/or abrasions with the fluid or tissues of aborted foetuses of diseased cattle can also be a source of the disease in humans. Work-related contact with the cattle or their product is the major risk for human brucellosis. Abattoir, farm, and laboratory workers, as well as veterinarians, are known risk groups for *Brucella* infection. *B. melitensis* biovar 1 has been isolated from the farmworkers' blood working as milkers in cattle farms (Osman et al. 2015).

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According to the notifiable diseases statistics published by the Ministry of Health, only a few human brucellosis cases (three in 2014; six in 2015; five in 2017; four in 2019) have been reported per year. According to the latest general census, the population in Northern Cyprus was estimated as 286 257 inhabitants. Although brucellosis is endemic in livestock, the rate of human cases is considered to be low. Since the use of raw and/or unpasteurised milk in dairy products and the consumption of raw meat and meat products are uncommon in the country, the transmission of brucellosis to humans is potentially reduced.

In livestock, herd identification of brucellosis generally relies on the presence of typical clinical manifestations, particularly abortion, serology and isolation of the causative agent. In any case, diagnosis of brucellosis in one or more infected animals is considered to be indicative of an infection in a population. Therefore, a prompt and accurate diagnosis of brucellosis is extremely crucial and important in the control and eradication of the infection in livestock flocks (Saavedra et al. 2019). Since there is not just a single test enabling the precise identification of *Brucella* organisms, a combination of growth traits, serological, bacteriological and molecular methods should be used for the accurate identification (OIE 2018). Despite some challenges, isolation of the organism from appropriate pathological specimens is still regarded as the gold standard diagnostic method for the laboratory diagnosis of brucellosis because of its specificity and suitability for biotyping of the isolate (Yu and Nielsen 2010; Asfaw Geresu and Mamo Kassa 2016). On the other hand, molecular methods, particularly PCR-based methods have increasingly and successfully been used in the diagnosis of the infection.

In the present study, in order to increase the isolation sensitivity of *Brucella* organisms from pathological specimens, both Farrell's medium (FM) and the CITA medium were used simultaneously as described earlier (De Miguel et al. 2011; De Nardi et al. 2015; Kolo et al. 2019). From 351 specimens, comprised of 32 aborted foetuses and 319 milk samples, a total of 109 *Brucella* strains (31.1%) comprising 19 strains (59.4%) from foetus samples and 90 strains (28.2%) from milk samples were isolated. The majority of the isolates obtained from the aborted foetuses by means of isolation numbers, were of sheep origin (12/19; 63.2%) in parallel with the sample size, while, with respect to the isolation rate, the majority was of cattle origin (4/5; 80%). On the other hand, the majority of the isolates obtained from the milk samples both by means of isolation numbers and rates, were of cattle origin (68/90; 75.6% and 68/190; 35.8%, respectively) (Tables 3 and 4). All the isolated strains were identified as *B. melitensis* according to the previously described criteria (OIE 2018; Saavedra et al. 2019; Shalaby et al. 2019). Identification of the isolates was further confirmed by using molecular methods. All 109 isolates were analysed by using a real-time PCR with the *Brucella* genus and *B. melitensis* species specific primers. Therefore, isolation of *B. melitensis* was documented in livestock in Northern Cyprus.

The results of the present study show compliance with previous studies carried out in Italy (De Massis et al. 2019), Egypt (Khan et al. 2019; Shalaby et al. 2019), Croatia (Spicic et al. 2010; Cvetnic et al. 2015), Albania (Fero et al. 2020), and Turkey (Sidamonidze et al. 2017), all indicating the isolation of *Brucella* species in the Mediterranean Basin, where the disease is endemic.

Following the preliminary identification, 27 selected isolates were further analysed with conventional methods as described earlier (OIE 2018; Saavedra et al. 2019; Shalaby et al. 2019) and 20 (74.1%) were biotyped as *B. melitensis* biovar 1 and seven (25.9%) were biotyped as *B. melitensis* biovar 3 (Table 5). No other *Brucella* species and/or biovars were detected throughout the study. Among the 20 *B. melitensis* biovar 1 isolates, three (15.0%), nine (45.0%), and eight (40.0%) strains were caprine, ovine, and bovine in origin, respectively. Similarly, among the seven *B. melitensis* biovar 3 isolates, one (14.3%), one (14.3%), and five (71.4%) strains were caprine, ovine, and bovine in origin, respectively. From the animal species point of view, four caprine strains were defined as three (75.0%) *B. melitensis* biovar 1 and one (25.0%) *B. melitensis* biovar 3, ten ovine strains were defined as nine (90.0%) *B. melitensis* biovar 1 and one (10.0%) *B. melitensis* biovar 3, and 13 bovine strains were defined as eight (61.5%) *B. melitensis* biovar 1 and five (38.5%) *B. melitensis* biovar 3 (Table 5).

These results are in agreement with previous studies showing the presence of *B. melitensis* biovars in livestock. In a study involving the analysis of *Brucella* field strains submitted for biotyping to the Italian National Reference Laboratory for

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Brucellosis from 2007 to 2015, De Massis et al. (2019) reported the presence of *B. abortus* biovars 1, 3 and 6 with a frequency of 90.1%, and *B. melitensis* biovar 3 with a frequency of 9.9% from a total of 2 981 strains isolated from cattle. On the other hand, the same study showed that *B. abortus* biovars 1 and 3 with a frequency of 4.3%, *B. melitensis* biovars 1 and 3 with a frequency of 95.3%, and *B. ovis* with a frequency of 0.4% were present among the 2 279 strains isolated from the sheep and goats. In contrast to the results of De Massis et al. (2019), neither *B. abortus* nor any other *Brucella* species were isolated in the present study. On the other hand, the isolation rates of *B. melitensis* biovar 1 and 3 from cattle and sheep showed similarities with slight differences.

Until now several multiplex PCR protocols, using different primer combinations, have been described for the identification of *Brucella* species (Sumathi et al. 2019). In 1994, a multiplex PCR protocol, called AMOS PCR, was established by Bricker and Halling (1994) to identify *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* at the species level by using five primers. More recently, another protocol, namely the Bruce-ladder multiplex PCR assay was defined by Lopez-Goni et al. (2008) to identify all *Brucella* sp. including the six terrestrial species and the marine species at the genus level, as well as the vaccine strains of *B. abortus* S19, RB51, and *B. melitensis* Rev 1 by using eight primers. Based on the Bruce-ladder multiplex PCR assay, an improved multiplex PCR was further developed by Mayer-Scholl et al. (2010) which differentiated all the recognised *Brucella* species, including *B. microti*, *B. inopinata*, *B. ceti* and *B. pinnipedialis*.

In this study, all 27 selected isolates that had been initially biotyped as *B. melitensis* biovar 1 and biovar 3 by the microbiological and serological methods were subjected to the Bruce-ladder multiplex PCR assay for further confirmation. According to the amplicon sizes of 1 682, 1 071, 794, 587, 450 and 152 bp, all the isolates tested including four caprine, ten ovine, and thirteen bovine origins were typed and confirmed as *B. melitensis* field strains. Detection of the amplicon of 1 071 bp in *B. melitensis* Ether strain and suspected isolates indicated that all the isolates tested were wild field strains. Additionally, due to the presence and absence of the 587 bp and 218 bp fragments, respectively, neither the *B. abortus* S19 nor *B. melitensis* Rev 1 vaccine strains were considered to be present among the isolates tested (Dadar et al. 2019; Sumathi et al. 2019; Tekle et al. 2019).

These results are in full agreement with the findings of Cvetnic et al. (2015) who stated the detection of 14 *B. melitensis* isolates out of 336 cattle, sheep and goat samples with the same amplicon sizes, among which seven isolates were recovered from sheep, six isolates from goats, and one isolate from cattle. In Croatia, *B. melitensis* biovar 3 was confirmed for the first time in cattle by Spicic et al. (2010). In the Bruce-ladder multiplex PCR, the expected sizes of the amplification products for *B. melitensis* were 1 682, 1 072, 794, 587, 450 and 152 bp. Similarly, upon detecting the same amplicons in the Bruce-ladder multiplex PCR, Sumathi et al. (2019) identified and confirmed five *Brucella* field isolates recovered from affected sheep and goat farms as *B. melitensis*. Although the 1 682 bp band size fragment commonly amplified by the majority of *B. melitensis* strains was not produced by the eight isolates investigated by Tekle et al. (2019), the authors concluded that all the strains were regarded as *B. melitensis* based on the results of the Bruce-ladder multiplex PCR assay with the amplification of the other five regular fragments, combined with the microbiological and biochemical characteristics of the isolates. In the present study, although the 1 682 bp fragment was not amplified by one isolate (Figure 3, Lane 19), it was still defined as *B. melitensis* according to the criteria outlined by Tekle et al. (2019). In a study, Dadar et al. (2019) indicated the presence of the *B. melitensis* Rev 1 vaccine strain determined by the Bruce-ladder multiplex PCR assay from aborted sheep foetuses. In contrast to Dadar et al. (2019), no vaccine strains were detected in the present study. This event was not surprising since vaccination against brucellosis in livestock has never been practiced in Northern Cyprus.

Although the *Brucella* species are generally considered to be host-specific, it is not very uncommon (unlikely) that cross-species infections can occur in animals under certain circumstances like sharing the same stables, pasture, and facilities, or due to the close proximity of the farms and the uncontrolled movement of infected animals (Morales-Estrada et al. 2012; Morales-Estrada et al. 2016; Kolo et al. 2019). Kolo et al. (2019) reported the presence of *B. melitensis* in cattle in South Africa for the first time by isolating seven strains from seropositive slaughtered cattle. Among these seven strains, five were biotyped as *B. abortus* biovar 1

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(*n* = 2), *B. melitensis* biovar 2 (*n* = 1) and *B. melitensis* biovar 3 (*n* = 2). In a brucellosis endemic region of Mexico, Morales-Estrada et al. (2016) defined cross-infections with *B. abortus*, *B. melitensis* and *B. suis* in sero-positive cows and goats by identifying three isolates as *B. abortus* biovar 1 each from cow and goat manure, three isolates as *B. melitensis* biovar 1 from cow manure and one isolate as *B. suis* biovar 1 from cow manure. In Croatia, while Spicic et al. (2010) reported the isolation of *B. melitensis* biovar 3 in cows for the first time, later on, Cvetnic et al. (2015) conducted microbiological analyses on 336 cattle, sheep and goat samples from 2009 to 2013 and concluded with the confirmation of *B. melitensis* in 14 (4.2%) samples, among which, seven isolates were recovered from sheep, six isolates from goats, and one isolate from cattle. Shalaby et al. (2019) reported the isolation of *B. melitensis* biovar 3 from the tissue specimens of cattle, such as the uterus and/or lymph nodes. Khan et al. (2019) identified 17 *B. melitensis* biovar 3 strains from the lymph nodes, milk and foetal abomasal contents of infected cattle.

Cattle can also be infected with *B. suis* and *B. melitensis* when they graze together with infected pigs, goats or sheep. Cross-species transmission might be a problem in mixed herding areas. *B. abortus* has been isolated from species other than cattle, including sheep, and the finding of *B. melitensis* in cattle has demonstrated the susceptibility of cattle for this species of *Brucella*. The isolation of *B. melitensis* from bovine milk and vaginal secretions indicates that the microbe can be transmitted by infected cattle. However, the prevalence of *B. melitensis* in cattle, and the importance of cattle in the transmission of this pathogen, has not been studied in much detail. In addition, the efficacy of vaccinations against *B. melitensis* infections in cattle has not been investigated. Although the Rev 1 vaccination of cattle is currently not recommended and may involve some risk, the use of this vaccine in cattle might be effective against *B. melitensis*. It is imperative to know the species of *Brucella* present in the infected mixed herds before vaccination is initiated (Smits 2013). A likelihood of cow-to-cow transmission of *B. melitensis*, isolated from aborted cows in farms that had never reared small ruminants has been reported in a study performed in Egypt. In this study, it was also detected that different genotypes of *B. melitensis* could be isolated from the same animal following a multilocus variable-number tandem-repeat analysis (MLVA) (Hegazy et al. 2022). Shedding of brucellae in the uterine discharge of culture positive/seronegative aborting cows is thought to be a serious problem resulting in the maintenance and further spread of infection. *B. melitensis* biovar 3 was identified in two out of five dairy cows after abortion (El-Diasty et al. 2018). In a study performed by using the MLVA-15 technique, most (13 out of 17) of the *Brucella* isolates from ruminants were found to be *B. melitensis* biovar 3, eight of which were isolated from different necropsy materials (lymph nodes, foetus, spleen) from cows in Egypt (Menshawy et al. 2014).

In the present study, while *B. abortus* was not isolated from any of the bovine specimens, in concordance with the above-mentioned studies, eigth *B. melitensis* biovar 1 and five *B. melitensis* biovar 3 strains were isolated with a frequency of 61.5% and 38.50%, respectively. This can be explained by the traditional nature and structure of livestock breeding in Northern Cyprus, in which different animal species co-exist closely, enabling cross-species infections as well as interruptions in maintaining the eradication of brucellosis in the country.

In this study, no research was conducted to determine the risk factors that play a role in the transmission and spread of *B. melitensis* in cattle. Therefore, only speculations can be made regarding the reasons why *B. melitensis* is common in cattle in Northern Cyprus. As has been mentioned earlier, one strong speculation could be the latest spread of *B. melitensis* that occurred due to the imported Damascus goats from Syria in 1960. Since then, because of the strict trading restrictions and the nature of farming, *B. melitensis* became persistent and transmitted to all the livestock including cattle.

The results of the present study revealed that brucellosis is prevalent in cattle, sheep and goats with *B. melitensis* biovars 1 and 3 as the circulating genotypes. It is, therefore, concluded that the awareness of animal holders about the economic and zoonotic impacts of brucellosis should be raised, and implementation of effective control strategies and appropriate preventive measures should strictly be continued. Further research will be required for a better understanding of the epidemiology of the infection in livestock animals in Northern Cyprus by molecular characterisation of the isolates with more sophisticated molecular tools, such as MLVA, multi locus sequence typing (MLST) and whole genome sequencing.

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Conflict of interest

The authors declare no conflict of interest.

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