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Aetiology of livestock fetal mortality in Mazandaran province, Iran

Afsaneh Amouei [1, 2, 3] , **Mehdi Sharif** [1, 3] , **Shahabeddin Sarvi** [1, 3] , **Ramin Bagheri Nejad** [4] , **Sargis A Aghayan** [5] , **Mohammad Bagher Hashemi-Soteh** [6] , **Azadeh Mizani** [1] , **Seyed Abdollah Hosseini** [1, 2, 3] , **Sara Gholami** [3] , **Alireza Sadeghi** [7] , **Mohammad Sarafrazi** [8] , **Ahmad Daryani** Corresp. [1, 3]

[1] Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran

[2] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran

[3] Parasitology and Mycology Department, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran

[4] Brucellosis Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Alborz, Iran

[5] Laboratory of Zoology, Research Institute of Biology, Yerevan State University, Yerevan, Yerevan, Armenia

[6] Department of Clinical Biochemistry and Genetics, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran

[7] Mazandaran Central Laboratory of Veterinary Organization, Medical Sciences, Sari, Mazandaran, Iran

[8] Mazandaran Provincial Veterinary Department, Medical Sciences, Sari, Mazandaran, Iran

Corresponding Author: Ahmad Daryani
Email address: daryanii@yahoo.com

In the farming industry, the productivity of livestock herds depends on the fertility efficiency of animals. The accurate diagnosis of a broad range of aetiological agents causing fetal death is often difficult. Our aim was to assess the prevalence rates of *Toxoplasma gondii*, *Neospora caninum*, and *Brucella* spp. infections in ruminant abortion using bacteriological culture and molecular techniques in Mazandaran Province, northern Iran. Samples were collected from 70 aborted sheep, goat, and cattle fetuses between September 2014 and December 2015. Necropsy was performed on all the received samples, and brain tissue and abomasal content were obtained from the aborted fetuses. Protozoan infections were detected by specific polymerase chain reaction (PCR) and bacterial agents using bacteriological examinations and PCR assay.Infectious pathogens were detected in 22 out of 70 (31.4%) examined fetuses. Moreover, *T. gondii, N. caninum*, and *B. melitensis* were verified in 13 (18.6%), 4 (5.7%), and 2 (2.85%) samples, respectively. Our results showed that infection with the mentioned pathogenic agents brings about considerable fetal mortality, which can be a major cause of economic loss.The listed pathogens could be considered important etiological agents of fetal loss in Mazandaran Province, for which appropriate control measures such as vaccination and biosecurity can be implemented to prevent infection and reduce reproductive loss in livestock farms.

Aetiology of livestock fetal mortality in Mazandaran province, Iran

Afsaneh Amouei [1,2,3], Mehdi Sharif [1,3], Shahabeddin Sarvi [1,3], Ramin Bagheri Nejad [4], Sargis A. Aghayan [5], Mohammad Bagher Hashemi-Soteh [6], Azadeh Mizani [1], Seyed Abdollah Hosseini [1,2,3], Sara Gholami [3], Alireza Sadeghi [7], Mohammad Sarafrazi [8], Ahmad Daryani [1,3*]

[1] Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran
[2] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran
[3] Department of Parasitology and Mycology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran
[4] Brucellosis Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Alborz, Iran
[5] Laboratory of Zoology, Research Institute of Biology, Yerevan State University, Yerevan, Yerevan Armenia
[6] Department of Clinical Biochemistry and Genetics, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran
[7] Mazandaran Central Laboratory of Veterinary Organization, Medical Sciences, Sari, Mazandaran, Iran
[8] Mazandaran Provincial Veterinary Department, Medical Sciences, Sari, Mazandaran, Iran

afsane.amoueia@yahoo.com, msharifmahdi@yahoo.com, shahabesarvi@yahoo.com, r.bagherinejad@rvsri.ac.ir, aghayan.sargis@ysu.am, hashemisoteh@gmail.com, azadeh.mizani@live.com, hosseini4030@gmail.com, gholami4030@gmail.com, sari.sadeghi@yahoo.com, sarafrazi_m@yahoo.com, daryanii@yahoo.com

Correspondence*: Ahmad Daryani, Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, PC 4847191971, Sari, Mazandaran, Iran. Tel: +98 09111538420; Fax: +98 11 33543249.

ABSTRACT

In the farming industry, the productivity of livestock herds depends on the fertility efficiency of animals. The accurate diagnosis of broad range of aetiological agents is often difficult. Our aim was to assess the occurrence of *Toxoplasma gondii*, *Neospora caninum* and *Brucella* spp. in ruminant abortion using bacteriological culture and molecular techniques in Mazandaran province, Northern Iran. Samples were collected from 70 aborted sheep, goat and cattle fetuses between September 2014 to December 2015. A necropsy was done on all received samples and brain tissue and abomasal content were obtained from aborted fetuses. Protozoal infections were detected by specific PCR and bacterial agents using bacteriological examinations and PCR assay. In total, infectious pathogens were detected in 22 out of 70 (31.4%) examined fetuses. Moreover, *T. gondii, N. caninum* and *B. melitensis* were verified in 13 (18.6%), 4 (5.7%) and 2 (2.85%), respectively. However, study results showed that infection with mentioned pathogenic agents occurs in these ruminants with high abortion rates which can be a major cause of economic losses. Therefore, listed pathogens could be considered as important aetiological agents in aborted fetuses and appropriate immunization or biosecurity to prevent infection could reduce reproductive losses of livestock in the Mazandaran province.

INTRODUCTION

The productivity of livestock herds depends substantially on their reproductive efficiency. High fetal mortality rate is a major cause of economic losses in the farming industry and a broad range of protozoa, bacteria and viruses are reported from ruminant farms. Therefore, the definitive diagnosis of abortifacient infectious agents is often difficult and should be done in specialized laboratories. Several causative pathogenic agents are considered as potential sources of zoonotic infections that are of veterinary and public health importance (Moeller Jr 2001).

Toxoplasma gondii and *Neospora caninum* are well known protozoa causing congenital infections related to abortion, neonatal mortality and necrotic lesions in the central nervous system (Müller et al. 1996). These parasites belong to the phylum Apicomplexa which are morphologically similar but have some structural, molecular and antigenic differences. Their life-cycles are also analogous with different definitive hosts, such as felids and canids in *T. gondii* and *N. caninum*, respectively. They have similar intermediate hosts counting a wide range of warm-blooded animals. *T. gondii* is also an important pathogen of humans and can be attributed to handling or consumption of raw or uncooked meat and milk (Hutchison 1965; Tenter et al. 2000). Neosporosis, caused by *N. caninum*, was first diagnosed in 1990 and was attributed as a leading cause of abortion in cattle (Wouda et al. 1997). Moreover *N. caninum* can occur less frequently in small ruminants and is associated with epizootic abortion (Moreno et al. 2012). Although antibodies against *N. caninum* were identified in humans, this protozoan has not been isolated from human tissues (Ibrahim et al. 2009; Lobato et al. 2006). Fetal injuries in brain tissue are similar to *T. gondii* and *N. caninum* infections and lesions may be sparse and not easily found. Nevertheless, diagnosis of these two coccidian parasites was much improved by the development of PCR tests (Bretagne et al. 1993; Yamage et al. 1996).

Brucella is the most important abortifacient bacterial agent with great economic importance in livestock in many areas of the world. Brucellosis is still a widespread zoonotic disease and some of the species of genus *Brucella* are pathogenic for human (Leyla et al. 2003). Abortion is the most important clinical sign of the disease in infected female animals which usually occurs during the late of pregnancy (Radostits et al. 2006). However, symptoms of the disease are mostly not pathognomonic so, accurate and direct diagnosis depends on bacteriological tests (Blasco 1992).

Despite reports showing implication of *Brucella* spp. in small ruminant's abortion (Behroozikhah et al. 2012), limited epidemiological information is available about current frequency of *Brucella* abortion in animal population in Iran.

The main objective of this study was to provide data about the occurrence of *T. gondii*, *N. caninum* and *Brucella* spp. in cases of ruminant abortion (sheep, goats and cattle) in Mazandaran province, Northern Iran.

MATERIALS AND METHODS

Study area

The study was carried out in Mazandaran province, near the Caspian Sea, in northern region of Iran, where the geographic and natural climatic conditions (temperature and humidity with an annual rainfall of 500 mm and an average temperature of 17 ^{0}C) are suitable for livestock production. Data regarding each farm history including epidemiological area code, abortions and results of serological surveys for brucellosis using tests such as Rose Bengal, Wright and 2ME were obtained by interview with the herder and by examination of computerized herd records at the Central Laboratory of the Department of Veterinary Medicine in the Mazandaran province.

Sample collection

Between September 2014 and December 2015, a total of 70 aborted fetuses (sheep, goats and cattle) were collected from the Department of Veterinary Medicine in the Mazandaran province. All investigations reported here were approved by the Ethics Committee of Mazandaran University of Medical Sciences (No. 1055). Data related to each animal was recorded using 3 independent variables (region, age and animal species).

A necropsy was done on all received aborted fetuses. Samples of brain tissue and abomasal content were obtained from aborted, stillborn or weak animals that died. Different parts of the brain (cortex, midbrain, medulla, and cerebellum) were subjected to examination and preserved in 70% ethanol until use for PCR detection of *T. gondii* and *N. caninum*.

Bacteriological examinations

For isolation of *Brucella* spp., samples of fetal stomach content from aborted fetuses were cultured using standard previously described procedures (14). Briefly, fresh specimens were cultivated onto *Brucella* medium base (OXOID, CM169B) containing *Brucella* Selective Supplement (OXOID, SR0383A) and 5% horse serum. The plates were incubated at 37 ^{0}C in an atmosphere with 10% CO_2. *Brucella* was recognized by colony morphology, growth, culture, staining and biochemical characteristics such as oxidase, urease. Species and biovar of isolated *Brucella* strains were determined by standard methods including CO_2 requirement, H_2S production, agglutination with mono-specific antisera, susceptibility to fuchsin and thionin dyes and lysis by Tb phage (Alton et al. 1988).

Samples were also inoculated onto Blood Agar containing 7% defibrinated sheep blood, MacConkey Agar, Eosin Methylene Blue Agar and *Salmonella* Agar for the isolation of other microorganisms.

DNA extraction

For detection of Toxoplasma and Neospora, DynaBio DNA extraction kit (Takapouzist co, Iran) was used to extract DNA from 20 mg brain tissue samples of all the aborted fetuses in accordance with the manufacturer's protocol.

DNA from *Brucella* isolates obtained in the study was prepared by boiling method. A loopful of cultured bacterial cells suspended in 200 µl of phosphate buffered saline (PBS) and boiled for 10 min. The suspension was then centrifuged at 12000 rpm for 5 min and the supernatant was used as template DNA.

The concentration of DNA was estimated by spectrophotometric analysis at A260/280 and extracted DNA was stored at -20 ^{0}C prior to PCR analysis.

PCR assay

Toxoplasma

Detection of *T. gondii* was carried out using amplification of a repetitive 529 bp DNA sequence (RE). This gene was selected as the target for PCR amplification and has been shown to be 200- to 300-fold more sensitive than other markers. The forward primer TOX-4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and the reverse primer TOX-5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') were used (Homan et al. 2000). The PCR reaction was amplified in a total volume of 25 µL containing 12.5 µl of commercial premix (Ampliqon,

Denmark), 1 µl of total DNA, 0.6 µl of each primer (10 pmol/µl) (BioNeer, Korea) and 10.3 µl of PCR H2O. The reaction mixture was performed in a thermocycler (BioRad C1000, USA) with minor modifications conditions: 93 ^{0}C for 5 min as initial denaturation followed by 30 cycles at 93 ^{0}C for 30 sec as denaturation, 55 ^{0}C for 30 sec as annealing, 72 ^{0}C for 30 sec as extension, and final extension at 72 ^{0}C for 5 min. A negative control (1 µl DDW instead of DNA) and a positive control (*T. gondii* DNA, Accession No: KT715444) were also acted in each reaction.

Neospora

For *N. caninum* molecular diagnosis, a fragment of the Nc-5gene has been designed nested PCR using Oligonucleotide primers Np21plus (5'-CCCAGTGCGTCCAATCCTGTAAC-3') and Np6plus (5'-CTCGCCAGTCCAACCTACGTCTTCT-3') (as the external primers pair) and Np6 (5'-CAGTCAACCTACGTCTTCT-3') and Np7 (5'-GGGTGAACCGAGGGAGTTG-3') (as the internal primers pair) (Hughes et al. 2006; Müller et al. 1996). All PCR reactions were performed the same way mentioned above for *T. gondii*. For the first round of PCR, the reaction mixtures were carried out in an automatic thermocycler (BioRad C1000, USA) under the following conditions: 94 ^{0}C for 5 min, followed by 40 cycles at 94 ^{0}C for 40 sec, 62 ^{0}C for 30 sec, 72 ^{0}C for 30 sec, and final extension at 72 ^{0}C for 10 min. The products of the first round were used as template for the second round of amplification which was conducted under the following thermocycling conditions: 94 ^{0}C for 4 min, followed by 30 cycles of 94 ^{0}C for 30 sec, 56 ^{0}C for 30 sec and 72 ^{0}C for 30 sec. A final extension step was continued for another 3 min at 72 ^{0}C. Samples with 1 µl DDW instead of DNA were used as negative controls and DNA of *N. caninum* (Accession No: KR106185) was considered as positive control.

Brucella

To confirm *Brucella* species isolated, a previously described multiplex PCR assay was applied (Bricker & Halling 1994). Specific oligonucleotide primers were used targeting IS711 insertion sequence in *Brucella melitensis* and *Brucella abortus* for molecular detection (*B. melitensis* primer: AAATCGCGTCCTTGCTGGTCTGA, *B. abortus* primer: GACGAACGGAATTTTTCCAATCCC and IS711 primer: TGCCGATCACTTAAGGGCCTTCAT) (18). The PCR amplification was carried out on 1 µl of genomic DNA (prepared freshly by boiling method as described above) with the following steps: 95 ^{0}C for 5 min as initial denaturation followed by 35 cycles at 95 ^{0}C for 75 sec as denaturation, 55.5 ^{0}C for 2 min as annealing, 72 ^{0}C for 2 min as extension, and final extension at 72 ^{0}C for 5 min. PCR mixture also contained 12.5 µl 2X master mix (Ampliqon, Denmark), 0.5 µl of each *B. abortus* and *B. melitensis* primers, 1 µl of IS711 primer (10 pmol/µl) and water up to a total volume of 25 µl. Also, 1 µl DDW instead of DNA (Blank) and 1 µl of *B. melitensis* strain 16 M and *B. abortus* strain 544 DNA (positive controls) were also included in each reaction.

Electrophoresis

Five microliters of the PCR products were run by electrophoresis through a 1% agarose gel (BioNeer, Korea) stained with Safe stain (0.5 µg/ml- Cina Gen Co, Iran) and visualized using a transilluminator (UVITEC).

Statistical analysis

Statistical analyses were estimated using the chi-square test and were performed with SPSS version 14.0 software (SPSS, Chicago, Illinois).

RESULTS

Findings of our study are summarized in Table 1. A total of 70 samples were collected from animals associated with aborted fetuses from fourteen counties in Mazandaran providence. The great majority of abortions were during the last months of gestation. There were 57 sheep, 4 goats and 9 cattle.

Protozoal infections were detected by specific PCR in 17 out of 70 (24.3%) examined fetuses (Fig. 1 and Fig. 2). Of the infected fetuses, 22.8% (13/57), 25% (1/4) and 33.3% (3/9) were ovine, caprine and bovine fetuses, respectively. The presence of *T. gondii* DNA was confirmed in 13 out of 70 fetuses (18.6%) and *N. caninum* DNA was detected in 4 out of 70 fetuses (5.7%) (Table 1).

Positive bacterial cultures were obtained from 5 fetuses, *Escherichia coli* (3 cases) and *Brucella* Spp. (2 cases). *Brucella* strains were identified as *Brucella melitensis* biovar 1 by conventional methods. Both *Brucella* isolates gave a band of about 700 bp by PCR specific for *B. melitensis* (Data for one isolate shown in Fig. 3).

DISCUSSIOND

Aborted fetuses results in heavy economic losses to the livestock industry in the world. Either infectious or non-infectious agents may cause fetal mortality and the definitive diagnosis of abortifacient agents is often difficult in pasture-reared ruminants. Because farmer only submit a few aborted fetuses for diagnosis and sometimes, fetuses are often autolyzed. Although this works provides important data on some of causes of ruminant abortion in the Mazandaran providence, it was estimated about 31.4% of the examined cases. The fetuses of central area showed higher positive level than the other areas and it seems that this region might be more contaminated (Table 2). Aetiology agents associated abortions reported in previous researches using the various diagnostic techniques (Campero et al. 2003; Kim et al. 2002; Moreno et al. 2012).

Since protozoan infections were present as abortifacient agents the range of diagnostic techniques have been applied, including serological, histopathology, immunohistochemistry, bioassay, cell culture and molecular assays. PCR method is considered as a specific sensitive technique used to diagnose parasite-specific DNA sequences (Wastling et al. 1993). We used a 200- to 300-fold repeated 529 bp fragment for the diagnosis of toxoplasmosis because of its high sensitivity and specificity (Homan et al. 2000). Also for evaluation of neosporosis, we selected a nested PCR technique based on the highly repeated Nc5 region with the sensitivity of detection 5-fold (Almeria et al. 2002). Current study indicates that protozoa infections are important causes of abortion in these animals. According to molecular examination of brain samples taken from aborted fetuses, in 13 cases (18.6%) and 4 cases (5.7%) of all investigated abortions were found *T. gondii* and *N. caninum*, respectively. In our knowledge, the highest frequency rates of *T. gondii* and *N. caninum* were reported in the aborted ovine and bovine fetuses, respectively (Table 1). These results are in agreement with another study in which majority of abortions of these parasites occurred in the mentioned animals (Kim et al. 2002; Masala et al. 2007). Our work showed lower prevalence of *N. caninum* than *T. gondii.* This may be associated with that *N. caninum* is considered as one of the most important causes of reproductive failure in cattle flocks (Dubey et al. 2007) where as in the present study majority of tested animals were sheep. Protozoa infections associated abortions are often reported in the literature. Compared to the results obtained by Habibi et al. they showed higher molecular prevalence rates of *T. gondii* (37.5% of ovine abortions and 22.7% of caprine abortions) than us (Samadi et al. 2010). Moreno et al. examined 74 ovine and 26 caprine fetuses for the presence of *N. caninum* and *T. gondii* DNA in Spain and showed that prevalence rates were 5.4% and 6.8% ovine abortions and 3.8% and 11.5% caprine abortions (Moreno et al. 2012). Also, Sagar et al. reported *T. gondii* DNA in 1 (<1%) of the 242 aborted

231 bovine fetuses and *N. caninum* DNA in 50 (21%) of them in Switzerland (Sager et al. 2001). These
232 differences may be influenced by geographical distribution and employed techniques in diagnosis
233 of infection. On the other hand, results of the present investigation indicate that samples were not
234 co-infected between *T. gondii* and *N. caninum* infections and more researches with on more
235 samples will be needed.

236 Furthermore, the presented fetuses have exhibited other problems of bacterial infectious agents
237 including *E. coli* and *B. melitensis*. In this study, two sheep exhibited *B. melitensis* –associated
238 abortions. This supported using both conventional bacteriological methods and molecular
239 techniques as the markers of *Brucella.* Thought, culture is considered as the gold standard test in
240 the laboratory diagnosis and provides the definitive diagnosis of brucellosis (Araj 2010),
241 application of PCR assays is specific and unable to differentiate between *Brucella* species and as
242 well as can be useful in speed of genotyping (Leyla et al. 2003). In our experiments, *B. melitensis*
243 strains were found in 2 (2.9%) of 70 examined cases from Babolsar (western origin) taken from
244 the herds that was according to serological examination (Rose Bengal and Wright-2ME), showing
245 high titers in this area. However, interpret of *Brucella* titers is difficult because may be regarding
246 mass vaccination and needs to be verified (Fekete et al. 1992). These figures are lower than those
247 previously reported, in which *Brucella* was detected in 20.86%, 31% and 34.56% of aborted sheep
248 fetuses in Iran, Turkey and Greece, respectively. Of course, our finding is in accordance with
249 these findings who reported *B. melitensis* strain as a dominant strain in the sheep fetuses
250 (Dehkordi et al. 2012; Leyla et al. 2003; Samadi et al. 2010). Also, *E. coli* was found in 3 sheep
251 fetuses. In our study, the lack of isolations of *Salmonella* spp. and fungal abortion suggest that
252 these bacterial and fungal may not be important as aetiological agents in aborted fetuses.

253 **CONCLUSIONS**

In conclusion, molecular detection system is a powerful method for the diagnosis of diseases. This investigation provides a valuable data for understanding the role of some causative agents of abortion in ruminant flocks in Mazandaran, Iran. Our results suggest that transplacental transmission of pathogen agents occurs in these animals. Further studies are necessary to investigate the high rates of organisms as causes of abortion in ruminants and to evaluate the resulting economic losses to the industry. Listed pathogen agents considered as potential sources of zoonosis that could be treated for medical, veterinary and public health. Finally, health education programs often can reduce the transmission of infection agents to humans and domestic animals and Also, appropriate immunization or biosecurity to prevent infection could reduce reproductive losses in livestock.

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Figure 1

Agarose gel (1.5%) stained showing amplicons of *Toxoplasmagondii*.

Lane M, 100 bp DNA marker; Lane 1, Positive control; Lane 2, Negative control; Lane 3-15, Positive samples; Lane 16, Negative sample.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

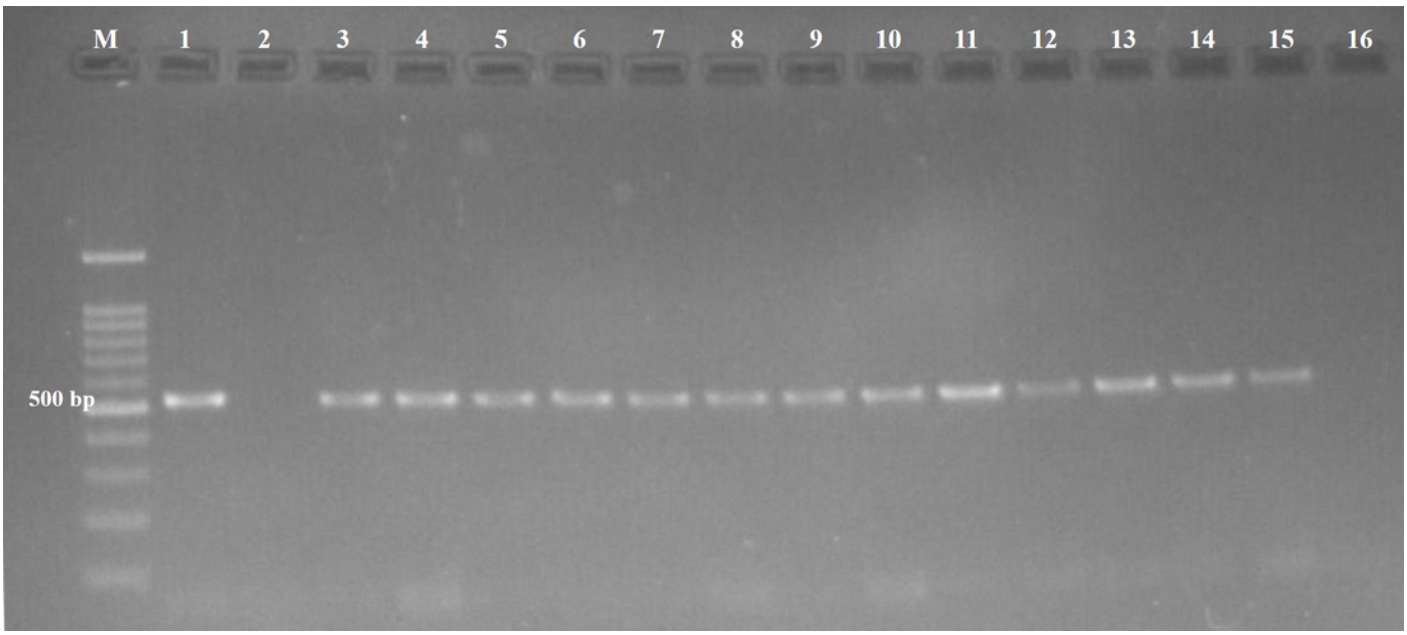

Figure 2

Examples of agarose gel electrophoresis of *Neospora caninum* obtained by nested-PCR.

Lane M, 100 bp DNA marker; Lane 1, Positive control; Lane 2, Negative control; Lane 3-6, Positive samples; Lane 7, Negative sample.

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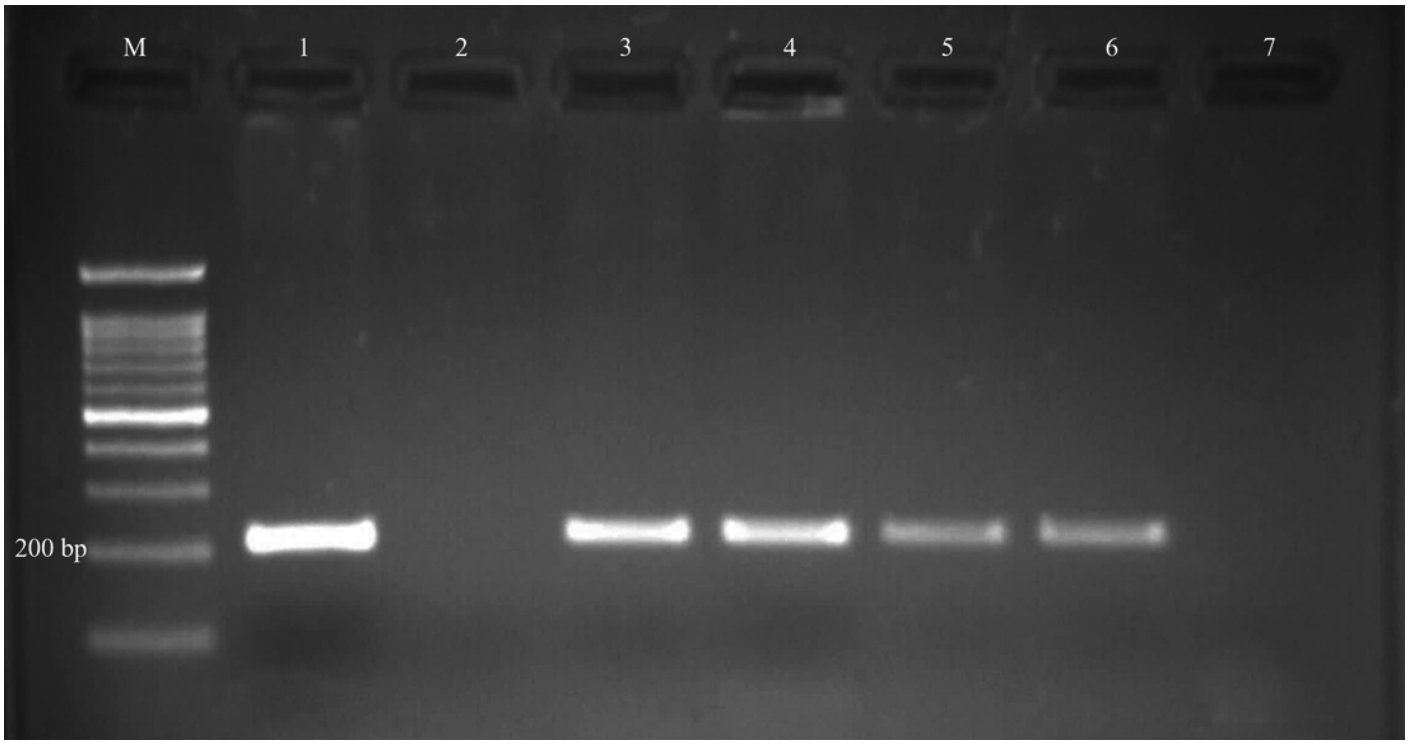

Figure 3

Examples of agarose gel electrophoresis of *Brucella* species PCR products using multiplex PCR.

Lane 1, *Brucella melitensis* strain 16M (as positive control); Lane 2, *Brucella abortus* strain 544 (as positive control); Lane 3, *Brucella melitensis* biovar 1 isolate; Lane 4, Negative control (without template DNA); Lane M, 100 bp DNA marker.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

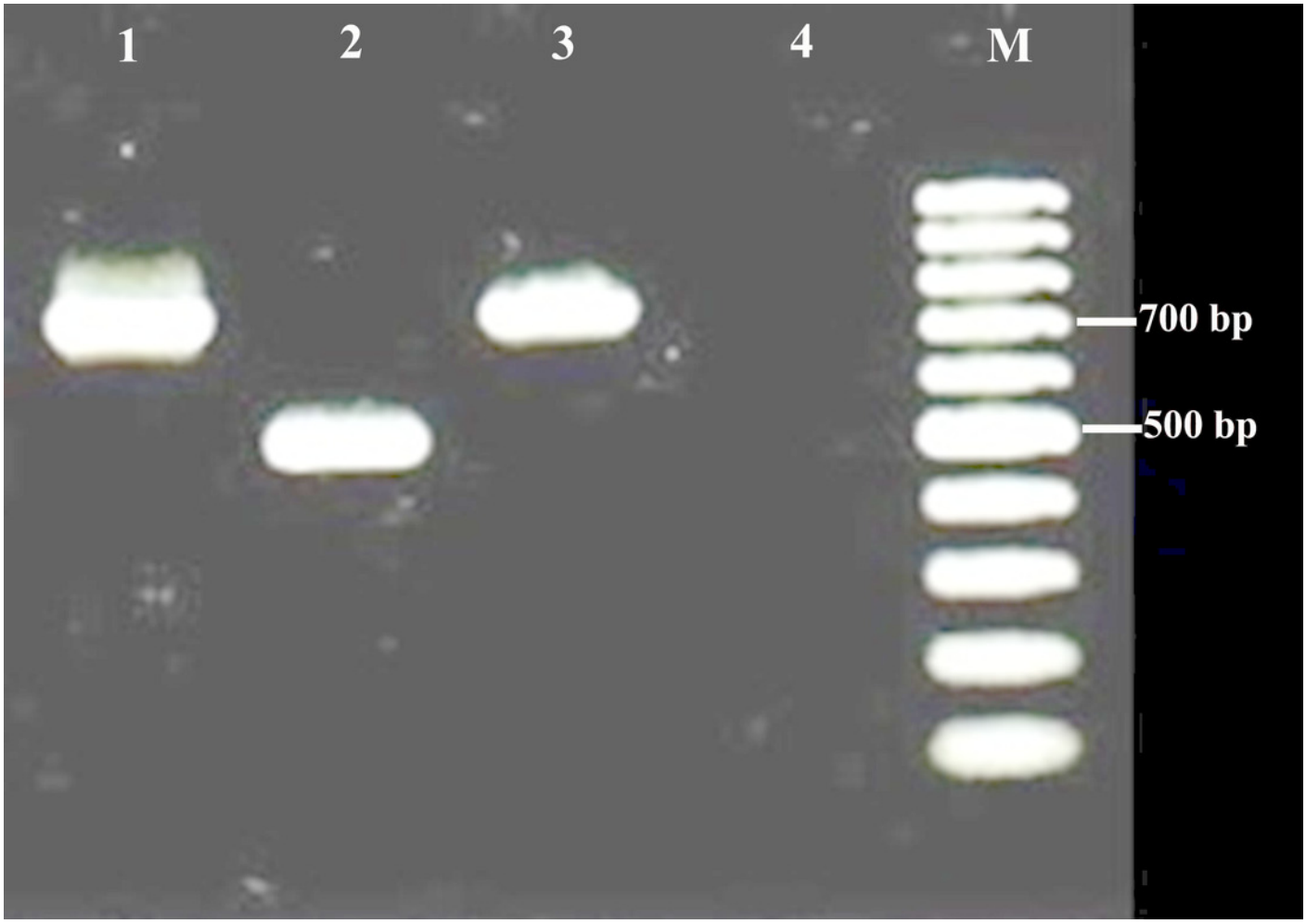

Table 1(on next page)

Summary of culture and PCR results obtained in samples submitted to infected fetuses

Species	No. samples	No. (%) of Identified infections				Total positive
		T. gondii by PCR	*N. caninum* by PCR	*B. melitensis* by culture and PCR	*E. coli* by culture	
Sheep	57	11 (19.3)	2 (3.5)	2 (3.5)	2 (3.5)	17 (29.8)
Goats	4	1 (25)	-	-	-	1 (25)
Cattle	9	1 (11.1)	2 (22.2)	-	1 (11.1)	4 (44.4)
Total	70	13 (18.6)	4 (5.7)	2 (2.85)	3 (4.3)	22 (31.4)

1

Table 2(on next page)

Summary of identified infections status in three areas from Mazandaran providence, Northern Iran.

∗ Variable which displays significant difference ($p < (0.05$ using Chi-square test.

1

Area	No. samples	No. positive of identified infections (%)				No. total positive (%)	OR	CI (95%)	P. value
		T. gondii	*N. caninum*	*B. melitensis*	*E. coli*				
East	16	1 (6.25)	1 (6.25)	0	0	2 (12.5)	1	-	-
Central	39	12 (30.8)	3 (7.7)	0	3 (7.7)	18 (46.2)	0.16	(0.01 - 0.9)	P= 0.02
West	15	0	0	2 (13.3)	0	2 (13.3)	0.9	(0.05 - 14)	P= 0.9