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Short Paper

Molecular detection of *Brucella melitensis*, *Coxiella burnetii* and *Salmonella abortusovis* in aborted fetuses of Baluchi sheep in Sistan region, south-eastern Iran

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Summary

Abortion in sheep and goats causes enormous economic losses. This study revealed the epidemiology of abortion caused by *Brucella melitensis*, *Coxiella burnetii* and *Salmonella abortusovis* in Baluchi sheep in Sistan region. In the autumn of 2015 and winter of 2016, a total of 78 aborted sheep fetuses were collected from all over the Sistan region. Risk factors, including location of livestock, history of abortion, gender of fetus, age of fetus, age of ewe and parity were obtained using a questionnaire. The results showed that 27 fetuses (35%) were infected with these organisms. Infection with *B. melitensis*, *C. burnetii* and *S. abortusovis* were identified respectively in 15 (19.2%), 13 (16.6%) and 1 (1.3%) fetus. Logistic regression analysis showed that infection with *B. melitensis* in male fetuses is higher than females (OR=3.73, P=0.040), also infection with *C. burnetii* in ≤ 2 years' ewes (OR=0.047, P=0.009) and 2-5 years' ewes (OR=0.197, P=0.069) is lower than ≥ 5 years' ewes.

Key words: Abortion, *Brucella melitensis*, *Coxiella burnetii*, *Salmonella abortusovis*, Sheep

Introduction

Abortion is a condition in which the pregnancy terminates before the fetus is viable (Boden and Andrews, 2015). Infectious and non-infectious multiple factors can cause spontaneous abortions. Infective abortions are caused by bacteria, fungi, protozoan and viruses. Non-infective factors are stress, housing conditions, transport, toxemia, metabolic disorders, inappropriate nutrition, hereditary factors, physical factors, etc (Beuzon *et al.*, 1997; Vidić *et al.*, 2007).

In the Sistan region, many people breed sheep and goats, much of the profit from sheep breeding belongs to lambing. Therefore, any disruption in this process can cause enormous economic losses. Importation of livestock from Pakistan, slaughtering of animals outside slaughterhouses and incomplete coverage of vaccination programs can cause spread of infectious disease among livestock in this region.

Brucella melitensis, *Campylobacter fetus*, *Salmonella abortusovis*, and *Chlamidophila abortus* are the most important pathogens involved in the abortion of ewes in Iran (Atyabi *et al.*, 2012). Some of the abortive microorganisms such as *Brucella* and *Coxiella* have been reported in the Sistan region (Esmaeili *et al.*, 2016). Thus, this study was conducted to investigate the prevalence of *B. abortus*, *C. brunetti* and *S. abortusovis* among aborted fetuses in the Sistan region.

Materials and Methods

Sampling methods

During the autumn of 2015 and winter of 2016, a total of 78 aborted Baluchi sheep fetuses were collected from all over the Sistan region. Risk factors including the location of livestock, history of abortion, gender of fetus, age of fetus, age of ewe and parity were obtained using a questionnaire. Age of the fetuses was estimated based on the crown rump length (Noakes *et al.*, 2001). Location of livestock was classified as eastern part of Sistan (Zahak and Hirmand counties), central part of Sistan (Zabol county) and western part of Sistan (Nimrooz and Hamoon counties).

Sheep owners were asked to transfer their aborted fetus to a nearby veterinary clinic. Biosecurity principles were taught to the sheep owners. Fetuses were placed in foam container on ice. Aborted fetuses were transferred to laboratory of anatomy of veterinary faculty within 24 h after abortion. After the autopsy, contents of abomasum and tissue of spleen were placed in sterile Eppendorf. Samples were stored at -20°C until use for DNA extraction.

The polymerase chain reaction (PCR)

Spleen and abomasum samples was used following the manufacturer's instructions of a commercial DNA extraction kit (DNPTM yield DNA purification Kit,

Cinnagen Biotech Co.[®], Karaj, Iran). The DNA extracts were used as a template for PCR. Primer design was done as using prior studies (Table 1). The PCR solutions were placed in the thermocycler (Eppendorf, Germany). Predenaturation was performed at 94°C for 4 min, following that, 30 thermal cycle was set as follows: 45 s of denaturation at 94°C, 1 min of primer annealing at 54°C, 59°C and 64°C, respectively for *B. melitensis*, *C. burnetii* and *S. abortusovis*, 1 min of primer extension at 72°C, after completing these cycles a final extension was performed at 72°C for 10 min. Positive control (*B. melitensis* ATCC 23457, *C. burnetii* ATCC VR-615, and *S. abortusovis* ATCC 31684) and negative control (sample without genomic DNA) were used in all reactions. PCR products were used for electrophoresis in a 2% buffer TAE (Tris Acetic Acid EDTA, MERCK, Germany).

Statistical method

In this study, when one or both the spleen and abomasum of a fetus was positive in PCR test, that fetus was considered as positive case. Since the dependent variable was a dichotomous variable, the multivariate logistic regression method was used for statistical analysis of the data. Before applying logistics regression, the relation between each independent variable with infection was analyzed using Chi-square test. When p-value in Chi-square test was less than 0.25, that variable was considered in the regression model.

Also, contamination of spleen and abomasum were compared using McNemar test. 95% confidence interval for prevalence of bacterial infection was calculated using binomial distribution. SPSS version 23 was used for statistical analysis. The significant level was considered $P < 0.05$.

Results

Among 78 aborted fetuses, 27 cases (35%) (95% CI: 24%-46%) were identified positive by PCR method. Infection with *B. melitensis*, *C. burnetii* and *S. abortusovis* was observed in 15 (19.2%), 13 (16.7%), and 1 (1.3%) fetuses, respectively (Figs. 1, 2 and 3). Among these fetuses, 2 cases (2.6%) were infected with both *B. melitensis* and *C. burnetii*.

The prevalence of each bacterium was calculated based on independent variables (Table 2). Because of the collinearity between parity and age of ewe, the parity was not considered in multivariate logistic regression

model. The results showed that relationship between gender of fetus and infection with *B. melitensis* was significant, also the relationship between age of ewe and infection with *C. burnetii* was significant (Table 3). There was no statistically significant relationship between other independent variables and infection of fetuses.

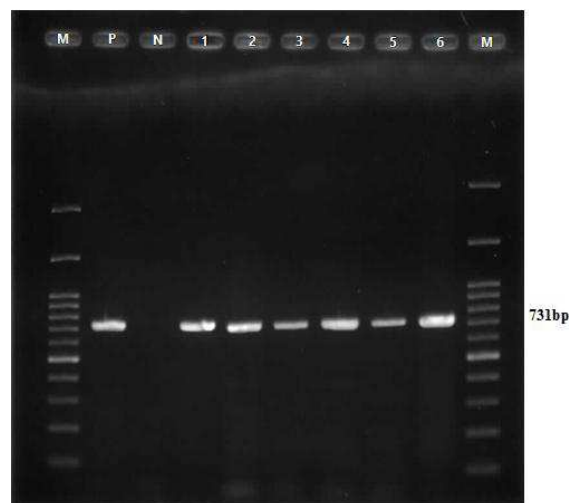


Fig. 1: PCR reactions for detection of *B. melitensis*. M: The 100 bp marker, P: The positive control, and N: The negative control. Positive samples (Lanes 1 to 6) characterized by identifying a gene fragment of 731 bp

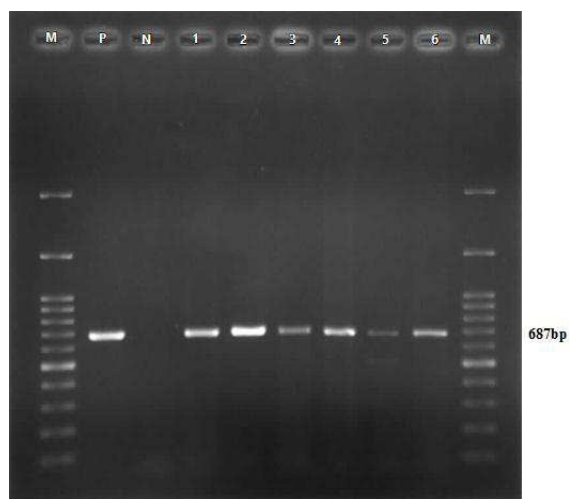


Fig. 2: PCR reactions or detection of *C. burnetii*. M: The 100 bp marker, P: The positive control, and N: The negative control. Positive samples (Lanes 1 to 6) characterized by identifying a gene fragment of 687 bp

Table 1: Characterization of primers using in this study

Gene	Primers	Length	Bacteria	Source
IS711	5'-AAA TCG CGT CCT TGC TGG TCT GA-3' 5'-TGC CGA TCA CTT AAG GGC CTT CAT-3'	731 bp	<i>B. melitensis</i>	Unver <i>et al.</i> , (2006)
IS1111	5'-TAT GTA TCC ACC GTA GCC AGT-3' 5'-CCC AAC AAC ACC TCC TTA TTC-3'	687 bp	<i>C. burnetii</i>	Parisi <i>et al.</i> , (2006)
IS200	5'-CGA TGA AAG CGT AAA TAA GG-3' 5'-TTC TCT TGT CAG TCT CAA AC-3'	900 bp	<i>S. abortusovis</i>	Belloy <i>et al.</i> , (2009)

Table 2: Prevalence of infection with *B. melitensis*, *C. burnetii* and *S. abortusovis* by independent variables in 87 aborted fetuses of Baluchi sheep in Sistan region

Category	Levels	No. of tested fetus	<i>B. melitensis</i>	<i>C. burnetii</i>	<i>S. abortusovis</i>
			No. (%) of infected fetus	No. (%) of infected fetus	No. (%) of infected fetus
Location of livestock	Eastern part	25	5 (20)	5 (20)	0 (0)
	Central part	38	6 (16)	5 (13)	1 (3)
	Western part	15	4 (27)	3 (20)	0 (0)
History of abortion	Yes	3	1 (33)	1 (33)	0 (0)
	No	75	14 (19)	12 (16)	1 (1)
Gender of fetus	Male	38	11 (29) ^a	7 (18)	0 (0)
	Female	40	4 (10)	6 (15)	1 (2)
Age of fetus	≤3 month	12	2 (17)	2 (17) ^a	0 (0)
	4 month	30	7 (23)	8 (27)	0 (0)
	5 month	36	6 (17)	3 (8)	1 (3)
Age of ewe	≤2 years	26	4 (15)	2 (8) ^a	0 (0)
	2-5 years	41	9 (22)	7 (17)	1 (2)
	≥5 years	11	2 (18)	4(36)	0 (0)
parity	First	25	5 (20)	4 (16)	0 (0)
	Second	23	2 (9)	4 (17)	0 (0)
	Third	18	5 (28)	2 (11)	1 (6)
	Forth ≥	12	3 (25)	3 (25)	0 (0)

^a Significant variables based on $P < 0.25$ that were considered in the regression model

Table 3: Multivariable logistic regression model for variables associated with fetuses' contamination in 87 aborted fetuses of Baluchi sheep in Sistan region

Infection	Independent variable	Levels	Odds Ratio	95% CI for OR	P-value
<i>B. melitensis</i>	Gender of fetus	Male	3.73	1.06-13.15	0.040
		Female	Reference category		
<i>C. burnetii</i>	Age of ewe	≤2 years	0.047	0.005-0.472	0.009
		2-5 years	0.197	0.028-1.143	0.069
		≥5 years	Reference category		

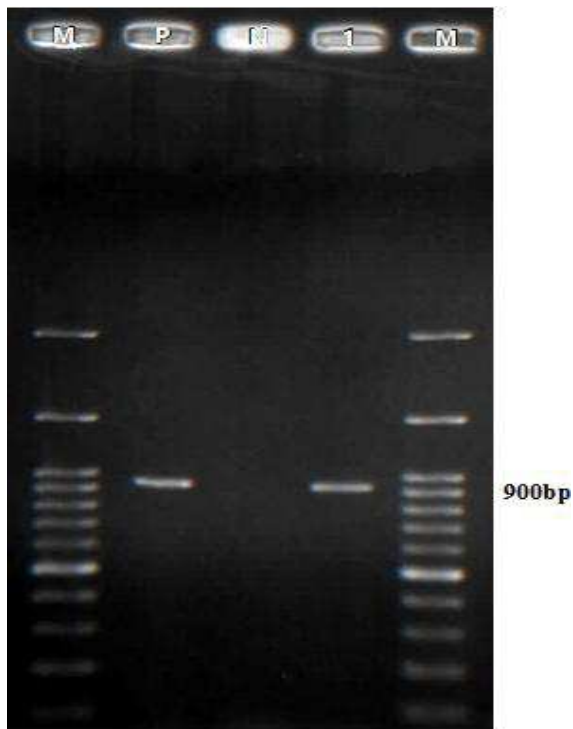


Fig. 3: PCR reactions for detection of *S. abortusovis*. M: The 100 bp marker, P: The positive control, and N: negative control. Positive sample (Lane 1) identifying characteristic gene fragment length of 900 bp

Among a total of 78 aborted fetuses, 7 spleens (9%) and 9 abomasa (12%) were infected with *B. melitensis* and 4 spleens (5%) and 11 abomasa (14%) were infected with *C. burnetii*. McNemar test showed that there was not a statistical difference between the detection rate of *B. melitensis* in spleen and in abomasum ($P=0.791$) also there was not a statistical difference between the detection rate of *C. burnetii* in spleen and in abomasum ($P=0.065$).

Discussion

In the present study, *B. melitensis* was the most prevalent bacteria among three bacterial agents which were investigated in the present study. In a study in Shiraz, 198 aborted fetuses of sheep were collected and bacterial culture used for detection of *Brucella* in which 22 fetuses (11%) were infected with *Brucella* (Firouzi, 2006). In another study conducted in Kalaleh and Gonbad Kavus (Golestan province), a total of 57 aborted sheep fetuses were collected and their abomasum contents were tested by PCR (Mohammadi, 2016). It was concluded that 10 fetuses (17.5%) were infected with *B. melitensis*. In another study among aborted sheep fetuses referred to Hamedan Provincial Veterinary Service, it was found that 5.3% of fetuses were infected with *Brucella* (Gharahkhani *et al.*, 2011).

Because of the abundance of rural and nomadic

populations and consumption of non-pasteurized dairy products, there is a high prevalence of brucellosis in the Sistan region (Sharifi Mood *et al.*, 2007; Khammar *et al.*, 2015). In another study from Sistan, 100 samples of cow's milk were tested by PCR, 7 samples of which were infected with *Brucella* (Noori Jangi, 2016).

According to the results, prevalence of *B. melitensis* in male fetuses was more than female ones. More research in this field is necessary to find out the cause of it.

In a study conducted in Sistan and Baluchestan province in 2011, 190 sera were collected from butchers and slaughterhouse workers and it was established that the seroprevalence of phase I and II of Q fever were 18.1% and 14.4%, respectively (Esmaili *et al.*, 2016). Although *C. burnetii* has previously been reported from the human population of Sistan and Baluchestan province, the present study, is the first report of *C. burnetii* from the livestock population in the Sistan region. In the present study 17% of aborted fetuses were infected with *C. burnetii*. Another study was conducted in sheep herds with a history of abortion, in different regions of Iran (Asadi *et al.*, 2013), and it was found that 19.5% of sheep have antibodies against *C. burnetii*. In a study on aborted fetuses in Italy (Masala *et al.*, 2007), 18.1% of fetuses were infected with *C. burnetii*. But in a similar study conducted in Hungary (Szeredi *et al.*, 2006), only 2% of aborted fetuses were infected with *C. burnetii*.

In the present study, the prevalence of *C. burnetii* was increased with increasing age of ewes. Such a trend has also been reported in other studies (García-Pérez *et al.*, 2009; Knobel *et al.*, 2013), it may be due to cumulative exposure to the pathogen from a young age (Knobel *et al.*, 2013).

In the present study, *S. abortusovis* was detected from abomasum of one fetus. Other researchers have reported that *S. abortusovis* is one of the major causes of abortion in sheep. A research that was carried out in Charmahal va Bakhtyari province showed that 30 fetuses out of 54 aborted sheep fetuses (55%) were infected with *S. abortusovis* (Sharifzadeh *et al.*, 2007). Belloy's study in Switzerland showed that 20 fetuses out of 24 aborted sheep fetuses (83%) were infected with *S. abortusovis* (Belloy *et al.*, 2009). Other *Salmonella* serotypes may be more prevalent than *S. abortusovis* in the Sistan region.

The present study showed that there is a high prevalence of *B. melitensis* and *C. burnetii* in aborted fetuses in the Sistan region. These bacteria caused not only economic losses due to abortion in livestock, but also the health of consumers of livestock products to be compromised.

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