Archives of Razi Institute, Vol. 74, No. 3 (2019) 295-301

Short Communication

A study on *Mycoplasma agalactiae* and *Chlamydophila abortus* in aborted ovine fetuses in Sistan and Baluchestan region, Iran

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Received 18 February 2018; Accepted 15 April 2018
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ABSTRACT

Abortion is one of the most important economic issues in sheep flocks. *Chlamydophila abortus* is an agent of enzootic abortions in sheep. *Mycoplasma agalactiae* is the main etiological agent of contagious agalactia, which can cause abortion in sheep. The aim of this study was to investigate the prevalence of *M. agalactiae* and *C. abortus* among aborted ovine fetuses in Sistan and Baluchestan, Iran. Sheep owners were asked to transfer their aborted fetuses to a nearby veterinary clinic; furthermore, they were taught biosecurity principles. A total of 78 aborted sheep fetuses were collected from all over Sistan region in the autumn of 2015 and winter of 2016. The samples were then transferred in ice to the Anatomy Laboratory of the Veterinary Faculty of Zabol University, Zabol, Iran. The spleen and abomasum contents of the fetuses were sampled under sterile and safe conditions. Polymerase chain reaction was used to detect *M. agalactiae* and *C. abortus.* The results showed that 24 (30.8%) cases were infected with *M. agalactiae*. However, infection with *C. abortus* was not detected in any fetuses. There was no statistically significant relationship between such independent variables as the location of livestock, history of abortion, fetal gender and age, age and parity of ewe, and fetal infection with *M. agalactiae*. The high incidence of Mycoplasma contamination in this study may be due to inappropriate biosecurity measures and lack of vaccination against agalactia in sheep herds in Sistan region.

Keywords: Abortion, *Mycoplasma agalactiae, Chlamydophila abortus*, Sheep

Une étude sur la prévalence de *Mycoplasma agalactiae* et *Chlamydophila abortus* chez des fœtus ovins avortés dans la région de Sistan et Baluchestan, en Iran

Résumé: L'avortement est l'un des problèmes économiques les plus importants chez les troupeaux de moutons. *Chlamydophila abortus* est une cause d'avortements enzootiques chez les moutons. *Mycoplasma agalactiae* est le principal agent étiologique de l'agalactie contagieuse, susceptible de provoquer un avortement chez les moutons. Le but de cette étude était d'étudier la prévalence de *M. agalactiae* et *C. abortus* chez les fœtus ovins avortés du Sistan et du Baluchestan en Iran. Il a été demandé aux propriétaires des moutons d'envoyer leurs fœtus avortés à une clinique vétérinaire située à proximité et les principes de biosécurité nécessaires leur ont été également enseignés. Un total de 78 fœtus de moutons avortés a été collecté dans toute la région du Sistan à l'automne 2015 et à l'hiver 2016. Les échantillons ont ensuite été transférés dans la glace au laboratoire d'anatomie de la faculté vétérinaire de l'université de Zābol, à Zābol, en Iran. Le contenu de la rate et de l'abomasum des fœtus a été échantillonné dans des conditions stériles et.de sécurité. La réaction en chaîne de la polymérase a été utilisée pour détecter *M. agalactiae* et *C. abortus*. Les résultats ont montré que 24 cas (30,8%)

étaient infectés par *M. agalactiae*. Cependant, l'infection à *C. abortus* n'a été détectée chez aucun fœtus. Il n'existait aucune relation statistiquement significative entre des variables indépendantes telles que la localisation du bétail, les antécédents d'avortement, le sexe et l'âge du fœtus, l'âge et la parité des brebis et l'infection fœtale à *M. agalactiae*. La forte incidence de contamination par *Mycoplasma* dans cette étude peut être due à des mesures de biosécurité inappropriées et à l'absence de vaccination contre l'agalactia dans les troupeaux de moutons de la région de Sistan.
Mots-clés: Avortement, *Mycoplasma agalactiae*, *Chlamydophila abortus*, Mouton

INTRODUCTION

Abortion is one of the most important economic issues in sheep flocks. This condition occurs as a result of infectious and non-infectious causes. Among infectious etiologies, bacterial agents play an important role in the incidence of this condition. Most of enzootic abortions in sheep flocks occur due to *Chlamydophila abortus* (Buckley et al., 2007), which is a species in Chlamydiaceae family of Chamidydiales order (Entrican et al., 2001). All Chlamydiae have a unique biphasic cycle, which contains an extracellular infectious form invading the host cells called elementary bodies, as well as an intracellular non-infectious form called reticular bodies. These bacteria are capable of developing into resistant forms which evade the host immune system and cause a range of disorders, including pneumonia, encephalomyelitis, keratoconjunctivitis, and abortion (Rodolakis and Yousef Mohamad, 2010). Symptoms do not manifest in all animals harboring Chlamydia (Rank and Yeruva, 2014). *Mycoplasma agalactiae* is the main etiological agent of contagious agalactia syndrome in sheep and goats, mainly characterized by mastitis, arthritis, keratoconjunctivitis, and occasionally reproductive disorders, such as abortion (Hegde et al., 2014). This species transmits from infected to healthy animals via oral, respiratory, and mammary routes. The microorganisms disseminate into the environment by means of ocular and nasal discharge, milk, feces, urine, and excretions from open joint lesions or male genitourinary tract (Madanat et al., 2001). *Mycoplasma*s are the smallest of the prokaryotes which have no cell walls and can survive in the absence of oxygen (Maniloff, 1983). Some strains of these bacteria live in various surfaces of animal bodies and have a slight ability to survive in the environment. There are specific binding proteins which help some strains in terms of attachment and pathogenicity. In Sistan and Baluchestan province, animal husbandry is one of the main occupations, especially in rural areas; therefore, abortion can cause a lot of economic loss in this region. With this background in mind, the present study was conducted to investigate the prevalence of *M. agalactiae* and *C. abortus* among aborted fetuses in Sistan region.

MATERIAL AND METHODS

A total of 78 aborted fetuses belonging to Baluchi sheep were obtained from the counties of Sistan region, including Zahak, Hirmand, Zabol, Nimrooz, and Hamoon. The ovine fetuses were collected in the breeding season in the autumn of 2015 and winter of 2016. After teaching the biosecurity principles to the sheep owners, they were asked to transfer the aborted fetuses of their flock to a nearby veterinary clinic. They were paid money for the delivery of each fetus to be encouraged to participate in the research. The diet of the studied livestock living in this area included soaked thorns, wheat flour, wheat bran, and hay. Aborted fetuses were transferred in ice to the Anatomy Laboratory of the Veterinary Faculty of Zabol University, Zabol, Iran. The age of the fetuses was estimated based on the crown-rump length. The spleen and abomasum content of the fetuses were sampled under sterile and safety conditions in the microbiology

laboratory. The obtained samples were kept at -20 °C until the DNA extraction phase. The spleen samples were carefully dissected using sterile blades before DNA extraction. Genomic DNA was extracted from the spleen and abomasum content samples using the DNP™ Kit, High yield DNA Purification Kit (CinnaGen., Tehran, Iran), according to the manufacturer's instructions. Briefly, 100 µl of each sample was mixed with 400 µl of lysis solution and homogenized for 20 sec, and insoluble materials were degraded. After adding a 300-µl precipitation solution, the mixture was subjected to centrifugation (12,000 g) for 10 min; subsequently, the tube was decanted. In the next stage, the sample was mixed with 1 ml wash buffer, centrifuged (12,000 g) for 5 min, and decanted again; afterward, it was dried at 65 °C for 5 min. Subsequently, 50 µl of solvent buffer was added to the dried pellets, which was then centrifuged at 65 °C for 5 min and 12,000 g for 30 sec. The extracted DNA in the solvent buffer was stored at -20 °C until performing PCR. The DNA quality was measured spectrophotometrically, and the samples with low concentrations (lower than 100 ng μL^{-1}) were excluded from the analysis. The DNA extracts were used as a template for PCR. A 15-µ PCR solution was prepared (including 1 ml of each primer [Table 1]) using 3 ml of distilled deionized water, 8 ml of Master Mix (Pishgam biotech co ©, Iran), and 2 ml of DNA extract. Subsequently, the PCR solutions were placed in the thermocycler (Eppendorf, Germany). Predenaturation was performed at 94 °C for 4 min. Afterward, 30 thermal cycles were set as follows: 45 sec of denaturation at 94°C, 1 min of primer annealing at 64 °C and 55 °C for *C. abortus* and *M. agalactiae,* respectively, and 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 samples (obtained from the Microbiology Department of Zabol University) and negative control samples (in which a DNA sample was replaced by sterile water) were used in all reactions. In order to evaluate target gene segments in the PCR reaction, 5 µl of the PCR products was loaded on agarose gel containing 2% buffer Tris Acetic Acid EDTA (MERCK, Germany); in addition, a 100-bp marker (Pishgam Biotech Co©, Iran) was loaded. Voltage and amperage of electrophoresis were set at 80 volts and 220 mA, respectively. After 75 min, the gel was stained with 10 mg/ml ethidium bromide (Pishgam Biotech Co©, Iran). Separated DNA fragments were observed under ultraviolet light using a Gel Documentation device (Cambridge, England). In this study, the location of livestock, history of abortion, fetal gender and age, and age and parity of ewe were considered as independent variables, whereas infections with *C. abortus* and *M. agalactiae* were considered as dependent variables. The associations between the independent and dependent variables were investigated using Pearson's Chi-square test, likelihood ratio Chi-square test, linear-by-linear association Chi-square test, and Fisher's exact test. When one or both samples of fetal spleen and abomasum were positive in the PCR test, the fetus was considered a positive case. Furthermore, 95% confidence interval was calculated for the prevalence of bacterial infection using binomial distribution. Prevalence of bacteria was compared between the spleen and abomasum samples using the McNemar's test. All statistical analysis was performed using SPSS (version 23.0; IBM Corp., Armonk, NY, USA) at a significance level of 5%.

RESULTS AND DISCUSSION

Among 78 aborted fetuses, 24 cases (30.8%; 95% CI=20.8-42.2%) were infected with *M. agalactiae* (Figure 1). However, infection with *C. abortus* was not observed in any fetuses. The aborted fetuses were collected from the counties of Zahak, Hirmand, Zabol, Nimrooz, and Hamoon with the frequency distributions of 13, 12, 38, 9, and 6, respectively. Location of livestock was classified into three parts, including the eastern part of Sistan (i.e., Zahak and Hirmand counties), the central part of Sistan (i.e., Zabol county),

and western part of Sistan (i.e., Nimrooz and Hamoon counties). The prevalence of *M. agalactiae* was estimated based on the independent variables (Table 2). There was no statistically significant relationship between the independent variables and fetal infection. Based on the results, 16 (21%) spleens and 11 (14%) abomasa were infected with *M. agalactiae*. The results of the McNemar's test showed no statistical difference in the rate of *M. agalactiae* in the spleen and abomasum samples (P=0.383). In this study, no *C. abortus* was found in aborted fetuses by PCR method. In a study performed by Chisu et al. (2013) in Sardina, Italia, the infection rate of *C. abortus* in aborted fetuses and placenta was reported as 6%. In Jordan, this rate was estimated at 63% in 2014 (Ababneh et al., 2014). However, in Egypt, infection with *C. abortus* was obtained as 42% (Osman et al., 2011). Additionally, the infection rate in Brazil in 2010 was reported as 21.5% (Pinheiro Junior et al., 2010). In a study carried out in Alborz province, Iran, in 2014, *C. abortus* was isolated from 37% of cases (Ebadi et al., 2015). Furthermore, in another investigation performed in the same year in Tehran, Lorestan, Qom, Fars, Bushehr, western Azerbaijan, and Khuzestan provinces, the infection rates were estimated at 37.7%, 32.9%, 30.3%, 30.3% 19.6% 17.5%, 15.6%, and 25.9%, respectively, with the overall prevalence of 25.6% (Esmaeili et al., 2015). In addition, in a study conducted in Chaharmahal and Bakhtiari province, Iran, the infection rate was measured at 52% (Mahzounieh and Pourahmad, 2014). Although *C. abortus* can be detected in the abomasum content and spleen of fetuses, *C. abortus* is more likely to be detected in the placenta (Saleh et al., 2013). Due to the nomadic life of most of the herders in Sistan region, sampling from placenta was not always possible. Nonuse of placenta sampling can be one of the reasons for not detecting *C. abortus* in this study. In addition, the difference between the prevalence of *C. abortus* obtained in the current research and other mentioned studies may be due to the specific geographic and climatic conditions of Sistan region. In this regard, wind of 120 days, dust storms, and scarcity of the pastures all affect the survival of some microorganisms in this region. *Mycoplasma agalactiae* is one of the organisms which is rarely detected or hard to be detected in affected flocks. The routine methods for the recognition of *Mycoplasma* in a herd are mainly based on classic ways, including biochemical tests and immunofluorescence. However, these methods are not only time-consuming but also difficult to interpret because of both false positive and negative responses through serum crossover reactions (Zendulkova et al., 2007). In addition to these methods, there are culture and PCR methods which have both advantages and disadvantages. Although the culture method is known as a standard method, it is time-consuming, expensive, and in some cases, its sensitivity is lower than that of PCR, which is due to dead or uncultivable organisms.

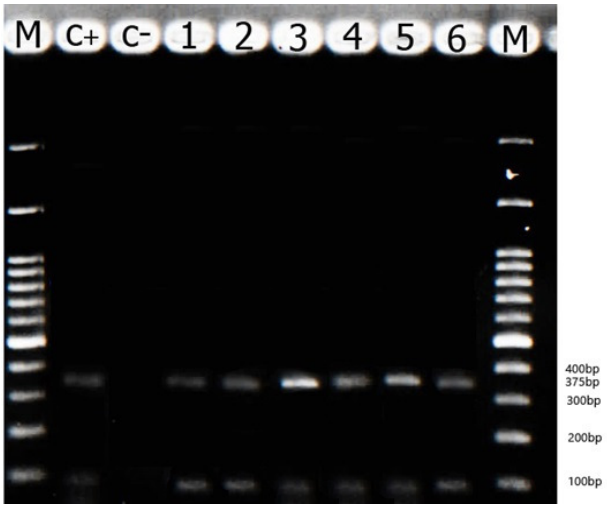

Figure 1. Polymerase chain reactions for the detection of *Mycoplasma agalactiae* (Positive samples (lanes 1 to 6) were characterized by identifying a gene fragment of 375 bp. N represents the negative control, P represents the positive control, and M represents the 100-bp marker.)

Table 1. Characterization of primers used in this study

Gene	Primers	Length	Bacteria	Source
16S rRNA	F:ATAATGACTTCGGTTGTTATT R:TGTTTTAGATGCCTAAACAT	127 bp	*C. abortus*	(Messmer et al., 1997)
16S rRNA	F:AAAGGTGCTTGAGAAATGGC R:GTTGCAGAAGAAAGTCCAATCA	375 bp	*M. agalactiae*	(Tola et al., 1997)

Table 2. Prevalence of infection with *M. agalactiae* in 87 fetuses by independent variables

Independent variable	Levels	No. of tested fetus	*Mycoplasma agalactiae*		P-value
			No. of infected fetus	Prevalence of infection	
Location of livestock	Eastern part	25	8	32%	0.574
	Central part	38	13	34%	
	Western part	15	3	20%	
History of abortion	Yes	3	1	31%	0.674
	No	75	23	33%	
Fetal gender	Male	38	12	32%	0.880
	Female	40	12	30%	
Fetal age	≤3 months	12	4	33%	0.253
	4 months	30	12	40%	
	5 months	36	8	22%	
Age of ewe	≤2 years	26	6	23%	0.843
	2 -5 years	41	16	39%	
	≥5 years	11	2	18%	
Parity of ewe	Primiparous	25	10	40%	0.225
	Multiparous	53	14	26%	

The PCR is a suitable and quick method for the diagnosis of infectious agents (Bashiruddin et al., 2005; Amores et al., 2010). To the best of our knowledge, no study has addressed sheep abortion due to *Mycoplasma* or investigated the abomasum content and spleen samples up to now. In previous studies, Belloy et al. (2003) separated *Mycoplasma* mostly from the joint and to a lesser extent from the eye swabs. In contrast, Zendulkova et al. (2007) isolated these species mostly from the eye swabs and to a lesser extent from the joint. In another study, Kheirabadi and Ebrahimi (2007) separated the majority of *Mycoplasma* from milk samples and to a lesser extent from joint samples. As the results of this study indicated, there were notable

Mycoplasma contaminated cases (30.7%) among aborted ovine fetuses in Sistan region. This may be due to the adoption of inappropriate biosecurity measures and lack of vaccination against agalactia in sheep herds in this region. The results revealed no significant difference in the rate of *M. agalactiae* in the spleen and abomasum. It was demonstrated that the spleen and abomasum contents can be suitable places for sampling from the aborted lambs suspected of *M. agalactiae*.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The present study was financially supported by Iran's Student Basij Organization. The authors would like to thank Dr. Hashem Zadeh, Dr. Mish Mast, Dr. Bakhshi, Dr. Zamanian, and Dr. Bameri, as well as the veterinary clinician in Sistan for their collaboration in collecting samples. We also appreciate Mr. Shahriari and the laboratory staff for their assistance in performing the PCR procedure.

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