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Original Article

Isolation and Detection of *Mycoplasma agalactiae* from Semen Samples of Goats

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ABSTRACT

Contagious agalactia (CA) is a highly infectious disease of goats and sheep, and is a form of Mycoplasmosis, which is usually enzootic. Since *Mycoplasma agalactiae (M. agalactiae*) is the main cause of this disease in goats, the aim of this study was to isolate and detect *M. agalactiae* from semen of goat bucks. Thirty-nine semen samples were collected from goat bulks, and all samples were cultured in PPLO broth medium supplemented for *M. agalaciae* isolation. The bacteria DNAs were extracted from clinical samples and the PCR assay was applied to detect *Mycoplasma* genus and *M. agalactiae* species using specific primers, which amplified a 163bp fragment in *16SrRNA* gene and a 375bp fragment in *lipoprotein* gene. The PCR evaluations were performed for both the clinical samples and the cultures. Out of the 39 samples, 29 (74.3%) of the cultures were shown positive and typical *Mycoplasma* colonies grew on PPLO agar, which could be considered as the diagnostic method. In addition, 38 (97.4%) samples had positive PCR results for *Mycoplasma* genus and six (15.3%) of the samples were shown to be positive using PCR for *M. agalactiae* as the diagnostic method. In the present study, *M. agalactiae* was detected in semen of goat bulks for the first time in Iran. Therefore, it is recommended to concern semen as one of the significant sources for this pathogen and the possibility for transmission to the female goats through semen is highlighted. Moreover, presence of this microorganism in semen could be involved in infertility of goat population.

Keywords: Goat buck, *Lipoprotein* gene, *Mycoplasma agalactiae*, Semen, *16SrRNA*

L'isolement et l'identification du *Mycoplasma agalactiae* à partir de sperme de bouc

Résumé: La maladie agalactie est l'une des maladies les plus infectieuses chez les moutons et les boucs. Cette maladie est dans la catégorie des infections à mycoplasme et est habituellement endémique. *Mycoplasma agalactiae* est la principale cause de cette maladie chez les boucs. Le but de cette étude était l'isolement et l'identification du *Mycoplasma agalactiae* à partir du sperme de bouc. Au total, 39 échantillons de sperme de boucs ont été prélevés. Une culture cellulaire a été effectuée sur tous les échantillons dans un milieu de culture adapté et spécifique (PPLO Broth) au *Mycoplasma agalactiae*. Après extraction de l'ADN des bactéries, des tests PCR ont été menés sur tous les échantillons en utilisant des amorces spécifiques à une séquences du gène 16S rRNA de 163 pb de longueur pour l'identification du genre *Mycoplasma* et une deuxième de 375 pb composant une partie du gène d'une lipoprotéine spécifique à l'espèces *Mycoplasma agalactiae*. Sur un total de 39 échantillons analysés, 29 échantillon (74.3%) étaient positifs dans le mileu de culture PPLO. Nos analyses par PCR spécifique au genre Mycoplasma ont révélé que 38 des 39 échantillons (97.4%) étaient positifs parmi lesquels 6 (15.3%) étaient également positifs au test PCR spécifique à l'espèce *Mycoplasma agalactiae*. Ce

travail représente la première étude sur l'isolement et l'identification du *Mycoplasma agalactiae* présent dans le sperme des boucs en Iran. Les résultats de cette étude ont montré que le sperme pourrait être un endroit approprié pour la présence de cet agent pathogène et sa transmission à la femelle. Par conséquent, ce facteur peut être considéré comme l'une des causes potentielles d'infertilité chez les chèvres.

Mots-clés: boucs, gène lipoprotéine, Mycoplasma agalactiae, sperme, 16s rRNA,

INTRODUCTION

Contagious agalactia (CA) syndrome is one of the most serious diseases affecting the small ruminants (Bergonier et al., 1997) and is the transmissible Mycoplsmosis of sheep and goats (Dedieu et al., 1995). The CA is an infectious diseases caused by several spices of *Mycoplasma* (de la Fe et al., 2009) and *Mycoplasma agalactiae* (*M. agalactiae*) is the historical agent of this syndrome in goats (Dedieu et al., 1995) in addition to *Mycoplasma mycoides subsp mycoides LC (Mmm LC), Mycoplasma capricolum subsp capricolum (Mcc)*, *Mycoplasma mycoides subsp capri (Mmc*) and *Mycoplasma putrefaciens (M. putrefaciens),* which all have been associated to the disease (Bergonier et al., 1997; Corrales et al., 2007). The CA has been reported in Southern Europe (Bergonier et al., 1997), South America, and North Africa (OIE., 2008). It is also regarded as a serious problem in Iran, where over 1300 cases of the disease were reported in 2006 (OIE., 2008). Several aspects of CA epidemiology have still remained unclear, such as the role of male animals with clinical symptoms and obvious lesions in disease transmission (Bergonier et al., 1997; de la Fe et al., 2010). Classic lesions have mostly been found in the mammary glands, joints, eyes, and respiratory tract (OIE., 2008), as well as the genital lesions, in which *Mycoplasma* could be involved (de la Fe et al., 2009). *M. agalactiae* affects the genital organs of the females, which might present as granular vulvovaginitis in goats (Lambert, 1987; Bergonier et al., 1997). This microorganism has been isolated from the semen of experimentally infected male sheep (Ak et al., 1995), and detection of this pathogen in addition to *Mmc* in the semen samples of goats has also been reported from Spain (de la Fe et al., 2009; Gomez-Martin et al., 2012). In another study, *Mmc* was shown to cause experimental ulcerative balanopsthitis and vulvovaginitis in sheep (de la Fe et al., 2009). Another species, *M. putrefaciens* was isolated from genital lesions of an adult male and several female goats during a clinical outbreak of CA with mixed infection of this spices and *M. agalactiae*. There are several reports of *Mycoplasmas* isolation from semen of horses and cattle, all of which have been associated with infertility and reproductive failure (Bielanski et al., 2000; Spergser et al., 2002). In cattle, presence of *Mycoplasma bovis (M. bovis)* has been reported in abortions and genital disorders (Byrne et al., 1999) as well as in cases of infertility and reduction of semen (Kissi et al., 1985; Nicholas and Ayling, 2003). According to the literature, Genital tract infection caused by *M. bovis* may also lead to infertility in male and female camels (Nicholas and Ayling, 2003; Abo-Elnaga et al., 2012). Breard and Poumarat (1988) managed to isolate *Mcc* from bull semen, and there are also reports of *M. agalactiae* isolation from the goat semen (de la Fe et al., 2010; Gomez-Martin et al., 2012). This can confirm the possibility of *Mycoplasma* presence in the semen of goat bucks and provide the first evidence of *Mmc* shedding in semen of the naturally infected goat bucks. In addition, Gill et al. in 2003 isolated *M. putrefaciens* from oviduct, uterus, and testes of goats. The purpose of the current study was to isolate *M. agalactiae* from the semen samples of goat bucks for the first time in Iran.

MATERIALS AND METHODS

Sampling and cultures. Thirty-nine semen samples were collected from 39 goat bucks of the Raini breed that reared in Kerman province of Iran, and did not

present any symptoms of CA. All these samples were analyzed by culture and polymerase chain reaction (PCR) to detect *M. agalactiae* as the main CA agent. Overall, 28 semen samples were placed in PPLO broth as the transport medium and were transferred to *Mycoplasma* reference laboratory on ice packs. None of the goats from which the samples were taken showed any clinical signs of CA. Primarily, the isolated specimens were diluted and filtered in fresh PPLO broth and were then inoculated on PPLO agar medium (BBL, Becton Dickinson and company, Cockeyville, Sparks, MD, USA). The inoculated agar and broth were both incubated at 37 °C in 50% Co_2 and 98% humidity. The broths were observed daily for signs of growth and the plates were checked for the typical appearance of *Mycoplasma* colonies (Bergonier et al., 1997). *M. agalactiae* reference strain (NCTC 10123) and PPPLO broth were used as positive and negative controls, respectively in this study.

DNA Extraction and PCR. DNA was extracted from samples using a method previously described by Kojima et al. (1997) with some modifications. Firstly, PCR detection of *Mycoplasma* genus was performed (Kojima et al., 1997), and then all the positive samples were analyzed by the PCR procedure specific for *M. agalactiae* (Tola et al., 1996). The PCR mix for *Mycoplasma* genus was a total volume of 25 µl per sample, containing 2.5 µl of 10X PCR buffer (Sinagen), 2 µl of 50 mM $MgCl_2$, 5 mM dNTPs, 10 pm of each primer(Table 2) and 0.5 U Taq DNA polymerase (Sinagen). Afterwards, 15.3 µl of deionized distilled water and 2 µl of template extracted DNA were added. For *M. agalactiae*, 5 µl of the DNA sample was incubated in 100 µl of the reaction solution, which consisted of 0.1 µM of each primer, 50 µM dNTP, 10 mM Tris/HCl at pH of 8.0, 4 mM $MgCl_2$, 50 mM KCL, and 1 U Taq DNA polymerase. The PCR assay for detecting the genus was conducted in a Gradient Mastercycler (Eppendorff, Germany) as follow: 7.5 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 1 min at 72 °C, with a final extension cycle of 5 min at 72 °C. Next, the PCR procedure for detecting *M. agalactiae* was conducted as 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 65 °C, with a final extension cycle of 10 min at 67 °C. The amplified products were visualized by UV illumination after electrophoresis (1% agarose gel in 1×Tris–acetic acid–EDTA (TAE) buffer) and ethidium bromide staining.

RESULTS

All the 39 samples were collected from the semen of goat bucks and tested simultaneously by culture, *Mycoplasma* genus PCR (MPCR) and *M. agalactiae* PCR (MAPCR). The culture results revealed 29 (74.3%) of the semen samples to be positive as typical *Mycoplasma* colonies, and 10 (15.7%) semen samples as negative.

Table 1. List of the primers used in PCR assay for genus *Mycoplasma* and *M. agalactiae*

PCR	Sequences	Reference
***Mycoplasma* Spp**	M1F: 5'-GCTGCGGTGAATACGTTCT-3' M3R: 5'-TCCCCACGTTCTCGTAGGG -3'	Kojima et al. (1997)
M. agalactiae	FS1: 5'-AAAGGTGCTTGAGAAATGGC-3' FS2: 5'-GTTGGCAGAAGAAAGTCCAATCA-3'	Tola et al. (1996)

In addition, 38 (97.4%) samples were confirmed as positive for *Mycoplasma* and one (2.6%) as negative by the PCR method. Furthermore, *M. agalactiae* was detected in six (15.3%) of the samples using the species specific PCR, and 33 (84.7%) of the samples were indicated to be negative by this PCR technique (Table 2). The *M. agalactiae* PCR product was 375 bp in length (Figure 1).

DISCUSSION

In the present study *M. agalactiae* was isolated from semen samples of the goat bucks and was identified by culture and PCR assay for the first time in Iran. Results

of the study showed that 97.4% of the semen samples were infected by *Mycoplasms*. Therefore, this study confirmed the high rate of *Mycoplasma* presence in semen samples by repeating the assays for the transfer PPLO broth and all the materials which were used for sampling as well as for keeping the semen samples. Hasso et al. (1993) managed to isolate *M. agalactiae* from testicular exudates of infected goats, and de la Fe et al. (2010) detected *M. galactiae* from the semen of goat bucks.

Table 2. Results of the cultures and PCR assays

Samples	Total sample (n)	Culture (n)		*M*PCR (n)		*M. agalactiae* PCR (n)	
		Positive	Negative	Positive	Negative	Positive	Negative
Semen	39	29	10	38	1	6	33

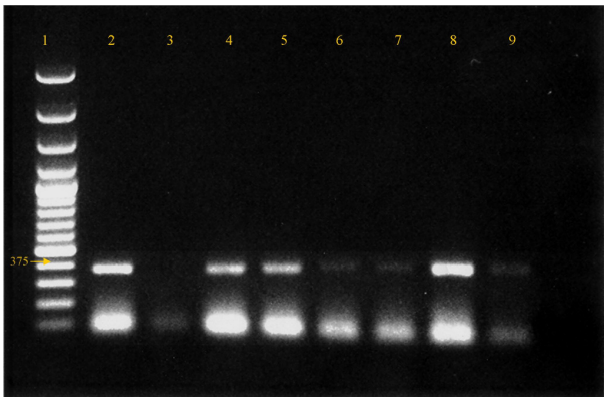

Figure1. *Mycoplasma agalactiae* PCR (MAPCR): PCR electrophoresis analysis in 1% gel agarose. *Lane 1* is the Marker (100bp DNA ladder), *Lane 2* is the positive control (375bp band, *Mycoplasma agalactiae*, NCTC 10123), *Lane 3* is the negative control (uncultured PPLO broth), and *Lanes 4 to 9* are the *Mycoplasma* isolates in this study.

Regarding the female animals, *M. agalactiae* has been isolated from vulvovaginal lesions in goats (Singh et al., 1974). As well, the semen samples of naturally infected bulls have been indicated to be positive for *Mmc* (Breard and Poumarat, 1988). In another evaluation, *M. putrefaciens* species was isolated from the testes of one goat buck (Gil et al., 2003). Gomez-Martin et al. (2012) have also reported isolation of *Mmm LC* from the semen of goat. Some of the viral and bacterial pathogens might lead in infertility directly by inducing different diseases of the reproductive tract or disorders associated with spermatozoa, which may prevent fertilization (Givens and Marley, 2008). The association of *Mollicutes* with ovine and caprine reproductive disorders has been mentioned less frequently (Kotani et al., 1980; Jones et al., 1983). *Mycoplasmas* are common inhabitants of genital mucosa in human and animals, and some species have been recognized as causes of reproductive failure and abortion (Bermudez et al., 1992; Miller et al., 1994). Genital mycoplasmosis may appear as inflammation of the genital tract but these microorganisms may also exist in the external genitalia without causing any clinical symptoms. The high prevalence of mycoplasmosis among sub-fertile and infertile cases could be an evidence of its potential role in male infertility ((Jurmanova and Sterbova, 1977; Le Grand et al., 1995; Nunez-Calonge et al., 1998). *M. bovis* and *Mmm LC* are the most important pathogens which were detected in the semen of cattle and camels and resulted in infertility (Bielanski et al., 2000; Abo-Elnaga et al., 2012). It has also been stated in the literature that *M. agalactiae* could have a remarkable part in flock infertility (Gil et al., 2003). Ak et al. (1995) experimentally inoculated *M. agalactiae* in semen of rams and observed a decrease in the volume, activity, motility, and concentration along with an increase in abnormal sperms. Results of the present study showed that 97.4% of our samples were positive in detection by the PCR specific for diagnosing Genus *Mycoplasma*; furthermore, 22% of them were diagnosed to be *M. agalactiae* by the PCR assay for this species. In contrast, De la Fe et al. worked on 147 samples and just found 2% of the animals as positive for *M. agalactiae*.

Therefore, the results of this study were in agreement with (de la Fe et al., 2009) regarding the investigation of *M. agalactiae* from semen of goat bucks, although the contamination rates were different which could be due to the smaller sample size in the current study. Gil et al. (2003) detected the both species of *M. agalactiae* and *M. putrefaciens* in a semen samples in goats and claimed that the samples had a mixed infection of *M. agalactiae* and *M. putrefaciens*. Their finding was in line with the present study regarding identification of *M. agalactiae*; however, *M. agalactiae* was more significant in semen samples of the goat bucks. It should be noted that the current study needs to fulfill more investigations in order to assess the presence other *Mycoplasma* species. The potential role of males as a permanent reservoir of *Mycoplasma* could result in infection of females via venereal transmission (Spergser et al., 2002; Sylla et al., 2005). In this study, *M. agalactiae* was isolated from the semen samples of some goat bucks without showing any signs of CA, which means that CA might be asymptomatic in these animals. As a result, the goat bucks could transmit the disease agent and the symptoms become apparent in the female goats after a while. Despite the limited isolation of *M. agalactiae* in the reports of de la Fe et al. (2010), they revealed a high number of male goats as *M. agalactiae* carriers, which along with the results of the present study could be of great significance since most of the infected males are asymptomatic. In conclusion, it was demonstrated in the current study that *M. agalactiae* might be shed in semen. Therefore, semen should be considered as an important source of contamination for this microorganism in goats, and it is of high importance since the infection might be asymptomatic in these animals. Furthermore, it is suggested to scrutinize the pathogenesis of *M. agalactiae* after mating in female goats in addition to assessing and determining the effects of this pathogen on infertility of both male and female goats. Moreover, it is necessary to verify the diagnosis of other *Mycoplasma* species, which were found to be positive in this study by PCR, and to identify the possibility of sexual transmission for all these species, which could lead in female genital diseases and infertility of the goat population.

Ethics

I 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.

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