Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Detection of *Mycoplasma ovipneumoniae* from Goats with Nasal Discharge by Culture and Polymerase Chain Reaction

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ARTICLE HISTORY

Received: December 27, 2010
Revised: January 24, 2011
Accepted: February 10, 2011

Key words:
Culture
Goat
Mycoplasma ovipneumoniae
PCR

ABSTRACT

In this study, the presence of *Mycoplasma ovipneumoniae* and other mycoplasmas was investigated by culture and PCR in the nasal swab samples of 692 goats with nasal discharges in 44 different flocks in eastern Turkey. The results showed that approximately 41% (18/44) of the flocks and 11% (75/692) of the goats harbored Mycoplasma agents. Among the Mycoplasma species; *M. ovipneumoniae* was the most prevalent species detected within 29.5% (13/44) of the flocks and 8.1% (56/692) of the goats. *Mycoplasma arginini* was also detected solely or in combination with *M. ovipneumoniae* in 18.2% (8/44) of the flocks and 1.9% (13/692) of the goats with nasal discharges. None of the samples were determined to be positive in the *M. mycoides* cluster and *Mycoplasma agalactiae* specific PCR analyses. The remaining 12 samples could not be identified at species level. Because the investigation of other agents which may cause respiratory problems such as *Mannheimia* was beyond the scope of this study, it is not possible to establish strong association between *M. ovipneumoniae* and disease. However, it may be logical to put forward that this agent might be the main cause of respiratory problems in at least three flocks in which the isolation rates of 50% or higher were obtained.

To Cite This Article: Ongor H, R Kalin and MN Acik, 2011. Detection of *Mycoplasma ovipneumoniae* from goats with nasal discharge by culture and polymerase chain reaction. Pak Vet J, 31(3): 244-248.

INTRODUCTION

Mycoplasmas are associated with a number of serious diseases of sheep and goats, most notable of which are contagious agalactia and caprine pleuropneumonias, especially contagious caprine pleuropneumonia, which lead to significant economic losses as a result of decreased milk production, morbidity and occasionally, mortality throughout mainland Europe, Asia and Africa (Nicholas, 2002).

Mycoplasma ovipneumoniae, first isolated in New Zealand in 1974, is considered as the cause of a non-progressive (atypical) pneumonia (Gilmour *et al*., 1979). Despite experimental reproduction of *M. ovipneumoniae* infection (Gilmour *et al*., 1979), and indications that this microorganism is associated with an increasing number of outbreaks, little is known about its role in respiratory diseases of small ruminants. In the USA, it has been implicated as the cause of a long-term coughing syndrome, with rectal prolapse (Niang *et al.,* 1998) and in New Zealand it has been linked to a chronic non-progressive pneumonia, prevalent in young sheep (Alley *et al.,* 1999).

It has been suggested that the interaction of *M. ovipneumoniae* with the host prevents normal ciliary activity, facilitating the invasion of the lower respiratory tract by other organisms, including *Mannheimia haemolytica* (Martin and Aitken, 2000). Ciliostasis and loss of cilia has been demonstrated in tracheal organ cultures (Jones *et al.,* 1985), which may lead to the inability to efficiently clear infectious agents from mucosal linings, promoting infection. Work carried out by Niang *et al*. (1998) detected a high level of ciliary autoantibodies associated with *M. ovipneumoniae* infection, which could induce inflammatory responses and loss of function.

Owing to the existence of high heterogeneity among *M. ovipneumoniae* isolates, it is possible that both pathogen and non-pathogen strains can be available in the same animal which may eventually cause variation in the morbidity and mortality of the disease due to this agent. Another disadvantage of high heterogeneity of the agent is

that immune response against one strain may not provide protection against diseases due to other *M. ovipneumoniae* strains (Ionas *et al.,* 1991; Parham *et al.,* 2006).

Studies on small ruminant mycoplasmas have mainly focused on contagious agalactia and contagious caprine pleuropneumonia in Turkey (Ozdemir and Turkaslan, 2003; Ozdemir *et al.,* 2005; Çetinkaya *et al*., 2009). On the other hand, only a few studies concerning *M. ovipneumoniae* have been conducted (Haziroglu *et al.,* 1996) and therefore there is a paucity of information about the potential of this agent.

The current study was conducted to investigate the presence of *M. ovipneumoniae* and other *Mycoplasma* species by culture and PCR in nasal swab samples of goats with nasal discharges.

MATERIALS AND METHODS

Sample collection and mycoplasma isolation

A total of 692 nasal swab samples were collected from 44 goat flocks which had nasal discharges in eastern Turkey. The nasal samples collected from goats were cultured on modified Hayflick's broths including ampicillin. The samples were cultured by serial dilution in modified Hayflick medium (PPLO [pleuropneumonia-like organisms] broth without crystal violet (21 g/l), 20% decomplemented horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 4 ml of phenol red solution 0.5 % (w/v), 4 ml of thallium acetate 5% (w/v), 0.04% ampicillin) and by streaking onto solid agar (PPLO agar) of the same medium, simultaneously. The broth media (not including phenol red solution) were solidified by adding 1% noble agar (Difco, Detroit, USA). The samples were diluted by five consecutive 10-fold dilutions. The swabs were added to the first broth medium tube (5 ml) and were mixed thoroughly. Then, 300 µl of broth was taken from each tube and mixed in a separate tube. The samples were incubated at 37°C in 5% CO_2 for seven to ten days.

The broths were checked daily and the samples with growth, indicated by color change in the broth cultures, were subcultured onto agar plates from the last tube with color change. The plates were checked daily for the appearance of colonies. The isolated colonies were inoculated into the stock suspension (50% broth / 50% horse serum) and were kept at -20°C.

DNA extraction and PCR

The broth cultures at the highest dilution showing color change were centrifuged at 12,000 g for 20 min. and the pellet was re-suspended in 300 µl of distilled water. Suspected mycoplasma colonies from solid agar (as indicated by a 'fried egg' appearance) were transferred into a microcentrifuge tube containing 300 µl distilled water. Both solid and broth suspensions were then treated with 300 µl TNES buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and proteinase K (200 µg/ml), and were kept at 56°C for 1 h. Suspensions were heated at 95°C for 10 min to inactivate proteinase K.

Two different PCR procedures were applied. In the first procedure, DNA samples were tested using genus-specific PCR for *Mycoplasma* species (Lierz *et al.,* 2007) and in the second, they were tested using specific PCR assays for the *Mycoplasma mycoides* cluster (Bashiruddin *et al.,* 1994), *M. ovipneuomoniae* (McAuliffe *et al.,* 2003), *M. arginini* (Timenetsky *et al.,* 2006) and *M. agalactiae* (Foddai *et al.,* 2005). The details of primer sequences used in the PCR analyses and the lengths of amplification products are given in Table 1. The PCRs were performed in a TC 512 Temperature Cycling System (Techne, Staffordshire, UK) in a reaction volume of 50 µl containing: 5 µl of 10 × PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM (NH4)2SO4, 0.1% Tween 20), 5 µl 25 mM MgCl2, 250 µM each deoxynucleotide triphosphate, 1.25 U Taq DNA polymerase (MBI Fermentas, St Leon-Rot, Germany), 20 pmol each primer (Iontek, Turkey) and 25 ng template DNA. The amplified products were detected by staining with ethidium bromide (0.5 mg/ml) after electrophoresis at 80 V for 2 h (7 V/cm) in 1.5% agarose gels.

RESULTS

Bacteriology and genus-specific PCR findings

In agar medium, growth was seen in six (0.9%) of 692 samples, all of which were identified as *Mycoplasma* sp. by genus-specific PCR. On the other hand, color changes were observed from 75 (10.8%) of the samples in broths. When the samples were subcultured onto solid agar, growth was seen in seven samples which were confirmed to be *Mycoplasma* sp. by genus-specific PCR (Fig. 1). This included six samples which already gave positive results in the direct cultivation onto agar base. DNA samples extracted from broth cultures, in which color change was detected, were also subjected to genus-specific PCR and positive results were obtained from all the 75 nasal swab samples. The difference between the broth and agar culture results was determined as statistically significant ($P<0.001$).

When the overall culture results were assessed at flock level, 18 (40.9%) of the 44 goat flocks were found positive for the presence of *Mycoplasma* sp. The isolation rate of *Mycoplasma* sp. was calculated as 22.5% (75/334), when the total number of goats within 18 flocks was considered.

Species-specific PCR findings

In the species-specific PCR analyses, 50 (7.2%) samples were identified as *M. ovipneumoniae*, seven (1.0%) as *M. arginini* and six (0.9%) samples were positive for both agents. None of the samples were found positive in the *Mycoplasma mycoides* cluster and *M. agalactiae* specific PCR analyses. The remaining 12 samples could not be identified at species level (Table 2).

In the species-specific PCR analysis of the six positive samples on agar medium, both *M. arginini* and *M. ovipneumoniae* were identified from four samples. On the other hand, one sample was positive for *M. arginini*, the remaining sample could not be identified at species level (Fig. 1).

DISCUSSION

The presence of *M. ovipneumoniae* and other mycoplasmas was investigated in the nasal swab samples of goats with nasal discharges in 44 different flocks in

Table 1: Primer sequences and lengths of PCR amplification products

Primer	Oligonucleotide sequences (5'-3')	Fragment Size (bp)	References
GPF	GCTGGCTGTGTGCCTAATACA	1013	Lierz *et al.* (2007)
MGSO	TGCACCATCTGTCACTCTGTTAACCTC		
MC323	TAGAGGTACTTTAGATACTCAAGG	1500	Bashiruddin *et al.* (1994)
MC358	GATATCTAAAGGTGATGGT		
LMFI	TGAACGGAATATGTTAGCTT	361	McAuliffe *et al.* (2003)
LMRI	GACTTCATCCTGCACTCTGT		
MAGF	GCATGGAATCGCATGATTCCT	545	Timenetsky *et al.* (2006)
GP4R	GGTGTTCTTCCTTATATCTACGC		
ma-mp1F	AGCAGCACAAAACTCGAGA	176	Foddai *et al.* (2005)
ma-mp1R	AACACCTGGATTGTTTGAGT		

Table 2: Culture and species-specific PCR results obtained from the nasal swab samples of goats with nasal discharges in eastern Turkey.

	Test	Number of positive flocks	Percentage of positive flocks	Number of positive samples	Percentage of positive samples
Culture + PCR*	Agar	3	6.8	6	0.9
	Broth	18	40.9	75	10.8
	M. ovipneumoniae	8	18.2	50	7.2
Species-specific PCR*	*M. arginini*	3	6.8	7	1.0
	M. ovipneumoniae + *M. arginini*	5	11.4	6	0.9
	Mycoplasma sp.	2	4.5	12**	1.7

*The differences between the broth and agar culture results and between the identification percentages of *Mycoplasma* species were statistically significant (P<0.001) **. These samples could not be identified at species level.

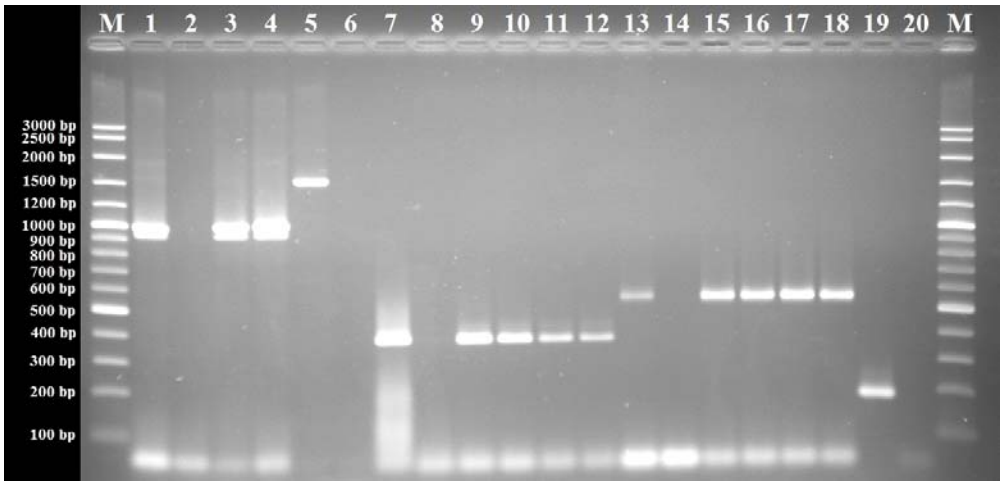

Fig. 1: Ethidium bromide stained agarose gel electrophoresis of the products amplified with PCR using the genus and species-spesific primers for Mycoplasma. M; 100 bp DNA ladder (vivantis), Lanes 1-4: Mycoplasma genus-specific PCR results: lane 1: *M. mycoides* subsp. *mycoides* SC positive control, lane 2: negative control, lanes 3-4: positive field samples. Lanes 5-20: Species-specific PCR results: lane 5: *M. mycoides* subsp. *mycoides* LC positive control, lane 6: negative control, lane 7: *M. ovipneuomoniae* positive control, lane 8: negative control, lanes 9-12: *M. ovipneumoniae* positive field samples, lane 13: *M. arginini* positive control, lane 14: negative control, lanes 15-18: *M. arginini* positive field samples. Lane 19: *M. agalactiae* positive control, lane 20: negative control.

eastern Turkey and the results showed that 41% of the flocks and 11% of the goats harbored *Mycoplasma* agents. Among the *Mycoplasma* species; *M. ovipneumoniae* was the most prevalent species detected within 29.5% (13/44) of the flocks and 8.1% (56/692) of the goats. The isolation rates of the agent varied from 8.6 to 57.1% by flock. In three flocks, *M. ovipneumoniae* was isolated from 50% or more of the goats with nasal discharges. Although this agent had been considered as saprophytic until recently, the regular isolation of *M. ovipneumoniae* from lesioned lung samples in many studies (Sheehan *et al.,* 2007; Çetinkaya *et al.,* 2009) and from outbreaks of atypical pneumonia cases (Gonçalves *et al.,* 2010) has renewed interest on the pathogenicity of this species.

It is now known that *M. ovipneumoniae* can cause respiratory disease in small ruminants by itself or may predispose animals to other pneumonia agents. In an abattoir based study, the results that *M. ovipneumoniae* has been detected in 90% of lungs with atypical pneumonia by culture and immunohistochemistry, while *Manheimia haemolytica* (*M. haemolytica*) and Parainfluenza-3 virus have been noted in only 30% indicate that *M. ovipneumoniae* can play major role in atypical pneumonia cases (Sheehan *et al.,* 2007). It has also been showed that *M. ovipneumoniae* may play

primary role in the fatal pneumonia cases due to *M. haemolytica* strains by weakening mucocilliary defence mechanism in the upper respiratory tract in sheep and goats (Niang *et al.,* 1998). The influence of *M. ovipneumoniae* in the rapid development of *Mannheimia* related pneumonia cases in small ruminants should therefore not be underestimated. In the UK, high morbidity and 20% mortality with post mortem lesions resembling contagious caprine pleuropneumonia have been reported in a goat flock which had mixed infection due to *M. ovipneumoniae* and *M. haemolytica* (Nicholas, 2002). In another study carried out in Portugal, *M. ovipneumoniae, M, arginini, M. haemolytica* and *Pasteurella multocida* have been detected in kids with severe respiratory signs and mortality rates of more than 20% have been noted (Gonçalves *et al.,* 2010).

Respiratory disease due to *M. ovipneumoniae* has also been reported in Spain, Nigeria and Sudan (Gonçalves *et al.,* 2010). In an experimental study conducted in Canada, the agent has been shown to be associated with the occurrence of pneumonia and fibrinous pleuritis (Goltz *et al.,* 1986). In the UK, *M. ovipneumoniae* has been detected in higher percentages from nasal swab and lung samples of sheep vaccinated against *Pasteurella,* but suffering from long-term coughing, when compared with those which were apparently healthy (McAuliffe *et al.,* 2003).

Because the investigation of other agents which may cause respiratory problems such as *Mannheimia* was beyond the scope of this study, it is not possible to suggest that *M. ovipneumoniae* was solely responsible for the respiratory problems within the eight flocks where only this agent was detected. However, it may be logical to put forward that this agent might be the main cause of respiratory problems in at least three flocks with the isolation rates of 50% or higher. In a study conducted in goats with pleuropneumonia, while *M. ovipneumoniae* has been isolated in 75% and 100% of nasal swab and lung samples, respectively, the other agents including the members of *M. mycoides cluster, Mannheimia* and *Pasteurella* could not be detected. The authors have therefore concluded that *M. ovipneumoniae* was responsible for the pleuropneumonia cases (Yang *et al.,* 2010).

None of the economically important *Mycoplasma* species, namely *M. agalactiae* and the members of *M. mycoides* cluster, was found in goats sampled in the present study. However, in a most recent study carried out in the same region, the causative agent of contagious caprine pleuropneumonia, *M. capricolum* subspecies *capripneumoniae*, has been reported in 37.5% of goats (Çetinkaya *et al.,* 2009).

Although a variety of growth media have been developed for the isolation of Mycoplasma species, there is still a lack of single medium with high sensitivity which was experienced in the current study. While PCR examination of broth cultures produced positive results in 75 samples, growth was observed from only six samples in the cultivation of samples onto agar medium. These results confirm the priority of PCR methodology over conventional culture in terms of processing contaminated materials. The overgrowth due to the other agents such as *Escherichia coli*, fungi and other microorganisms might have hindered the growth of *M. ovipneumoniae* which grows at a lower rate than the mentioned agents. Another plausible explanation for the difference between the broth and agar cultures can be that because *M. ovipneumoniae* isolates usually lack typical 'fried-egg' colony morphology, they might have been overlooked in agar cultures (Goltz *et al.,* 1986). The fact that *M. ovipneumoniae* could not be detected on its own in the species-specific PCR analysis of the six samples, which showed growth on the agar medium, support this opinion.

In this study, *M. arginini* was also detected solely or in combination with *M. ovipneumoniae* in 18.2% of the flocks and 1.9% of the goats with nasal discharges. The isolation rate of *M. arginini* was statistically lower than that of *M. ovipneumoniae*. This is in agreement with the current knowledge about the agent, as there is little evidence to connect *M. arginini* primarily with respiratory disease. However, its presence with major pathogens may increase pathological damage.

Mycoplasma ovipneumoniae strains have been determined to show high variability within and between flocks (Ionas *et al.,* 1993; Parham *et al.,* 2006). The co-existence of both pathogen and non-pathogen strains in the same animal can cause problems for diagnosis, establishing experimental infections and in vaccine preparations. Large scale molecular epidemiological studies are therefore required to determine the exact role of *M. ovipneumoniae* in atypical pneumonia cases and virulence differences of the strains. This will reveal predominant strains which may be used in the preparation of combined vaccines including the other agents, like *Mannheimia* and *Pasteurella*.

Acknowledgements

The authors wish to thank Dr. R. A. Nicholas for critical reading of the manuscript.

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