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Full Article

Isolation and identification of *Mycoplasma agalactiae* by culture and Polymerase Chain Reaction in Sheep and Goat Milk Samples in Kordestan province, Iran

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ABSTRACT

Contagious agalactiae (C.A.) is one of the most common disease affecting small ruminants which is caused by *Mycoplasma agalactiae*. This disease is particularly widespread around the world and Iran is one of the countries that C.A. is present. The aim of this study was isolation and identification of *M. agalactiae* (MG) with culture and PCR technique in milk samples in Kordestan province, Iran. A total of 367 milk samples were collected from sheep and goat. Specific published primers amplify a 375 bp gene of MG were used for PCR. Twenty (5.5%) out of 367 were positive in PPLO agar and 5 (25%) out of these isolates were positive with *Mycoplasma agalactiae* primers. Four (75%) out of 5 isolates was from sheep and 1(25%) from goat. Result of PCR with 367 milk samples showed that 11(3%) of them were positive with these primers. The isolation of *M. agalactiae* showed that C.A is present in Kordestan province and our results suggested that PCR method because of reduces the time consuming could be an alternative method beside culture.

Keywords: *Mycoplasma agalactiae*, Culture, PCR, Sheep, Goat

INTRODUCTION

Contagious agalactiae of sheep and goats is a serious infectious disease affecting small ruminants. It is characterized by arthritis, keratoconjunctivitis, and pneumonia, in females, mastitis and occasional abortion. *Mycoplasma agalactiae* is still regarded, particularly in sheep, as the "classical" a etiological agent of contagious agalactiae (Bergonier *et al* 1997). *M. agalactiae* primarily affects the mammary gland, eyes, and joints (Nicolas 2002). The disease is rapidly spread by contact between infected and healthy animals. Domestic sheep and goats of both sexes can be infected at the same frequency (Mdanat *et al* 2001), but morbidity is most often associated with pregnant and lactating females rather than males (Ruffin 2001). Contagious agalactiae is at present an important disease in countries with intensive small animal husbandry, such as Asia (Kusiluka *et al* 2000). The economic impact of the disease lies in the decrease or loss of milk production and, less often, also in abortions in pregnant dams. In the countries where sheep and goat dairy products are important foods as well as commercial commodities, contagious agalactiae is a serious problem in terms of veterinary

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health and socio-economic impacts (Nicholas 1998). Asymptomatically infected animals can shed mycoplasma for many years after infection, therefore, they play a very important role in the epidemiology of contagious agalactiae, making unsuccessful both prophylaxis and eradication programs. Most researchers use culture and molecular methods for isolation and identification of mycoplasma (Azvedo *et al* 2007, Momani *et al* 2006, Pirali & Ebrahimi 2007, Verbisck-Bucker *et al* 2008). De La Fe et al. (2005) reported a microbiological survey for mycoplasma spp. in Spain. There was a total of 38.5% positive flocks from which 37 mycoplasma isolates were obtained. *Mycoplasma* were isolated from 21 milk samples, *M. agalactiae* was isolated from 40% of the positive herds (27% of all isolations). Contreras et al. (2008) cultured 1068 bulk-tank goat milk samples. They showed that 84(7.9%) were positive for the presence of *Mycoplasma* species. Most of the species isolated were *M. agalactiae* (82%). Borry et al. (1963) reported the presence of agalactiae disease in sheep and goats in Iran for the first time. Sotoodehnia et al. (1986) cultured 490 sheep and goats milk. Ninety six isolates were biochemical identified as *M. agalactiae* of which only 23 were confirmed by serological test. There was not any isolation from milk samples were taken from Kordestan province. Because of their importance in veterinary medicine, and since infection spreads quickly once it is established in a herd, it is very important that specific and rapid diagnostic procedures are developed for their detections (Foddai *et al* 2005, Greco *et al* 2001). PCR has been accepted as a valuable method for diagnosis of mycoplasma infections using 16srRNA and also *Mycoplasma agalactiae PG2,* P80 predicted lipoprotein (Razin, 1994). It's noteworthy that culture is gold standard and must be noticed besides molecular methods. The aim of this study is detection and identification of *Mycoplasma agalactiae* in sheep and goat milk by culture and PCR in Kordestan province.

MATERIALS AND METHODS

Collection of milk sample. About 10 ml of milk samples from 271 sheep and 96 goats with or without sign of mastitis were obtained aseptically and collected into sterile tubes in Kordestan province. The samples were transported on ice to the Department of Microbiology in Razi Vaccine Serum & Research Institute, Karaj, Iran.

Mycoplasma isolation and identification. The milk samples immediately were cultured into the PPLO broth and on PPLO agar media following standard procedures. The inoculated media were incubated in a 5% CO_2 humidified incubator at 37 $^{\circ}$C. The broths were examined daily for signs of growth, such as change of pH indicated by a color change or turbidity change in the media. The plates were examined after 2–3 days under 100X magnification for the presence of a typical "fried egg" appearance of mycoplasma colonies (Kizil and Ozdemir, 2006).

DNA extraction. DNA was extracted from milk samples by the method described by Tola *et al,* (1997) with some modification as follows: At first 5 ml of milk sample was centrifuged for 10 min at 13000 g. The pellet was washed with mycoplasma wash solution (Rodwell & Whitecomb., 1983). The pellet suspended in 1ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) containing 0.5% sodium dodecyl sulphate to which was added 3 µl of proteinase K (20 mg/ml) and incubated for 3 hrs at 37° C. The lysate was extracted twice with 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1). Take the supernatant and add 400 µl of chloroform: isoamyl alcohol (24:1). Repeat the above step. Take the supernatant. The DNA was precipitated by adding 1 ml of 2-Propanole to the tube. After 5 min centrifugation, the DNA was washed with 70% ethanol, dried, and resuspended in 100 µl of TE buffer. The yield and the purity of the DNA preparation were determined by spectrophotometric analysis at 260 and 280 nm. Five micro liter of extracted DNA template was used in the PCR amplifications. DNA extraction from positive culture was done by using DNA kit

(CinnaGen Inc, Tehran, Iran) according to the manufacturer,s instruction.

PCR systems. *M. agalactiae*- specific amplification primer set FS1 5′-AAAGGTGCTTGAGAAATGGC-3′ and FS2 5′-GTTGCAGAAGAAAGTCCAATCA-3′ used that described by Tola et al (1997). Three micro liter DNA sample was incubated in a 50 µl reaction volume containing 4 µl (each primer), 2 µl 10mM dNTP mix, 1.5 µl 50mM $MgCl_2$, 5 µl 10X PCR buffer, 1 µl Taq DNA polymerase, 29.5 µl H_2O. PCR was performed in a eppendorf thermal cycler by using 1 cycle at 94 ° C for 5 min, 35 cycles of denaturation for 1 min at 94 ° C, annealing for 1 min at 55 ° C, extension for 1 min at 72 ° C and 1 extension cycle at 72 ° C for 10 min. The size of the amplified DNA fragment was 375bp. Bands were visualized after electrophoresis of 10 µl of the reaction mixture in a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). *M. agalactiae* vaccine strain and *M. pneumonia* were use as positive and negative controls.

Sequencing. The PCR product of 20 isolates send to MWG Company (Germany) for sequencing to confirm the PCR result.

PCR specificity and sensitivity. Specificity of PCR assay was checked by using *M. agalactiae* vaccine strain, *M. pneumonia* , *E.coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* DNAs. The sensitivity of PCR assay was evaluated by the 10-fold serial dilutions for the PCR were made by adding 10µl *M. agalactiae* stock culture to 90 µl sterile PBS.

RESULTS

Isolation and identification of *M. agalactiae*. Total of 367 milk specimens that collected from sheep and goat were analyzed. Twenty (5.5%) out of 367 were positive in PPLO agar. All isolates had typical colonies for mycoplasma. 16(80%) out of 20 isolated mycoplasma were from sheep and the others were from goat.

PCR. The PCR showed that 5(25%) out of 20 isolated mycoplasma were positive. Result of PCR with 367 milk samples with *M. agalactiae* primers showed that 11(3%) of them were positive. The 20 isolates of mycoplasma were subjected to PCR proceduer using primers FS1 and FS2, and then the 20 PCR products were sequenced. DNA sequences BLAST showed that 5 out of 20 which is 25% of isolated mycoplasma from culture were positive. Analysis of these results showed that, these primers amplyfies 328 to 330 bp whitin a 2166 bp fragment of *M. agalactiae* genome, and this fragment is located between base 586516 up to 588681 of 877438 bp circular DNA of this bacteria, which codes for a 721 amino acid fragment of *Mycoplasma agalactiae PG2,* P80 predicted lipoprotein, this fragment could be seen under "IPR004984 Mycoplasma_lipoprotein_cen_dom "accession in UniProtKB. This domain is found along with a C-terminal domain (IPR004890) in a group of Mycoplasma lipoproteins of unknown function. Also bioinformatics analysis of each of this 5 DNA sequences, when blasted using NCBI BLAST system, showed that one part of each of these 5 sequences are located at the beginning of P80 predicted lipoprotein which have 92-97% coverage with this protein. Another part of each of these 5 sequences are located upstream. One of the sequences submitted to GenBank (accession number HQ722028). If the gene subjected to analysis is a conserve gene then this differences could show polymorphism. Figure 1 showes PCR of milk samples.

Specificity and sensitivity. The 375bp fragment was seen with *M. agalactiae* vaccine strain. No fragments were obtained with other bacteria. PCR sensitivity was determined with culture of *M. agalactiae*. The detection limit was 10^2 CFU/ml.

DISCUSSION

The isolation of mycoplasmas is considered to be one of the most difficult tasks for diagnostic laboratories due to their inability to grow easily in

laboratory media in spite of the great improvement in medium formulations. The samples of choice for mycoplasma cultivation are milk, nasal secretions, joint and pleural fluid depending on the clinical disease (Momani *et al* 2006).

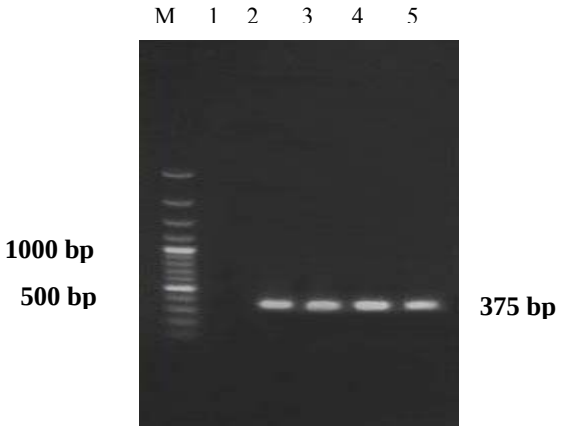

Figure 1. PCR amplification of milk samples by the *M. agalactiae* primers. M, molecular size marker (100bp); Lane 1, negative control (*M. pneumoniae*); Lane 2, positive control (*M. agalactiae* vaccine strain); Lane 3-5, milk samples.

In this study we showed that 20 (5.5%) out of 367 milk samples were positive in PPLO agar plate. Similarly, in one study in Turkey 17 isolates of *M. agalactiae* was obtained from 47 milk samples collected from the sick goats (Kizil & Ozdemir 2006). Azvedo et al. (2006) cultured 11 milk samples of caprine and reported that 4 of milk samples had *M.agalactiae*. Verbisck-Bucker et al. (2008) work on Spanish Ibexes. *M. agalactiae* was isolated from 46(11.2%) of the 411 samples. This study showed that wild population of animals must be considered for transmission of *M.agalactiae*. In Northern Jordan milk and nasal swabs were collected. Mycoplasmas were isolated from 17 (26%) of the 62 milk samples and 12 (3.9%) of the 310 nasal swab samples collected from goats and from 8 (13%) of the 62 milk samples and 7 (2.3%) of the 310 nasal swabs collected from sheep (Momani *et al.,* 2006). Sotoodehnia et al. (1986) cultured 490 sheep and goats milk. Ninety six isolates were biochemical identified as *M. agalactiae* of which only 23 were confirmed by serological test. There was not any isolation from milk samples were taken from Kordestan province.

PCR assay is routinely used in many laboratories and are extremely sensitive. It can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive. PCR has been accepted as a valuable tool for the diagnosis of mycoplasma infections and has the advantage of easy use, rapid availability of results, and standardization and is more suitable for processing of a large number of specimens (Foddai *et al* 2005). In the present study we showed that 5(25%) out of 20 isolated mycoplasma from milk were positive with *M. agalactiae* primers. Result of PCR with 367 milk samples with these primers showed that 11(3%) of them were positive.

Momani et al. (2006) isolated 44 mycoplasma that identified by newly developed PCR based molecular technique,denaturing gradient gel electrophoresis. One out of 44 isolates was *M. agalactiae*. Pirali and Ebrahimi (2007) with using of PCR method showed that 8 out of 47(17%) milk samples were positive with *M. agalactiae* primers in west central, Iran. Several PCRs specific for *M. agalactiae* have been developed and showed similar levels of sensitivity, although they are based on different gene sequences (Bashirudin *et al* 2005, Dedieu *et al* 1995, Subramaniam *et al* 1998, Tola *et al* 1997). These can be used directly on nasal, conjunctival, synovial and tissue samples; and have been used on milk samples where they have been reported to be more sensitive than culture (Tola *et al* 1997). PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24 hour enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicolas 2002). In Conclusion culture was the most sensitive test and followed by PCR as a rapid and economic tool for diagnosis of contagious agalactia using milk samples. The isolation of *M. agalactiae* showed that Contagious agalactiae is present in Kordestan province and our results suggested that PCR method because of reduces

the time consuming could be an alternative method beside culture.

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