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Research Article

IDENTIFICATION OF MYCOPLASMA AGALACTIAE IN MILK BY CULTURE AND PCR METHODS IN HAMEDAN OF IRAN

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ABSTRACT
Mycoplasma agalactia is responsible for several diseases in ruminants. These diseases include mastitis, arthritis, keratic, etc. Ruminants can have this infectious disease in the form of less severe and acute. Ruminants infections with this bacteria, causes milk loss and other sings and problems. Considering failure in treatment, presence of infection control techniques in herds can be beneficial and solve most of the problems, therefore, separation and recognition of the bacteria together with a fast and reliable method, like PCR, makes it possible and useful to prevent economic losses. Considering vaccination to control the spread of agalactia which is caused by these bacteria, the new separation strains can be used in new strains vaccine preparation. In this study tests were carried out on 367 milk samples from goats and sheep by culture and PCR techniques (considering *Mycoplasma agalactia*). In culture technique from 367 milk samples, 20 (5.4%) were tested positive. Also PCR results on 20 *Mycoplasma agalactia* samples separated by FS-1, and FS-2 primers, showed that five cases (1.3%) from *M. agalactia* consideration were tested positive. PCR results on 367 milk samples with primer, *M. agalactia* in 11 cases (3%) were tested positive. Results have shown using PCR techniques for a rapid and early identification and also a direct detection of *M. agalactia* bears a special importance. Although, it has been suggested that culture technique should also be used alongside PCR technique as a gold standard technique.

Keywords*: Mycoplasma Agalactiae, Milk, Hamedan*

INTRODUTION
Mycoplasmas were previously known as PPLO. These small polymorphic organisms are only between 126 and 300 nanometers and easily pass through filters of diameter 450 nanometers (Alain and Glen, 2005). These bacteria are generally negative-gram, move less and spore less. However, some of them have sliding motions in liquid environments. All of them are generally facultative anaerobic except for Mycoplasma pneumonia that is exceptionally aerobic. Mycoplasmas instead of a cell wall have a cell membrane consisting of three layers and containing amphipathic lipids like phospholipids, glycolipids, estriol, and proteins, thus they lack the DAP (diaminopimelic acid) existing in cell walls (Al-Momani *et al.*, 2006). Electron microscope investigations have shown only three organelles in these microorganisms, including cell membrane, ribosome, and prokaryotic genome, and no evidence of intracellular membranous structures like mesosome has been seen (Bridr´e. and Donatien, 2005; Arabi and Naser, 2010)

Mycoplasma Agalactiae
It was in 1923 that, for the first time, Brider and Donattien differentiated Mycoplasma agalactiae as the second known species of Mycoplasmas (Arabi, 2006). In fact, this bacterium was named by Wroblewski as Anulomyces agalaxi in 1931 (Arabi and Soyoodehnia, 1984). This species has a very smaller genome of 1×10^9 KD in comparison to other Mycoplasmas and in 1957, according to the new taxonomy, its name was changed to Mycoplasma agalactiae by Freundi. In Iran, about 30 years ago, Dr. Kaveh and Delpi from the Razi Institute reported a case of disease that was similar to Contagious Bovin Pelur Penomoni and was found among the herds of the north of Iran. The first test that should be applied to the collected samples is digitonin sensitivity test so that Mycoplasmas can be distinguished from Acholaplasmas. Mycoplasma agalactiae has surface proteins known as Vpmas on its surface that it plays a significant role in the dispersion of the disease. Similar to many pathogenic Mycoplasmas, the antigenic diversity of the

surface protein of agalactiae plays a very important role in the survival and dispersion of the bacteria inside the body of the host. The incubation period of the disease is 5 to 7 days under experimental conditions while it is longer and about 3 to 11 days under natural conditions. The very initial sign of the disease is a sever fever that does not last for long as well as considerable anorexia and disquiet. At first, milk secretion decreases, then pus comes out of the udder and finally, due to mammary gland fibrosis, milk completely dries up. In male animals this disease appears in form of arthritis, as its most prominent sign, and in female animals is accompanied by serene milk decrease and abortion. Mastitis usually appears in 2 to 3 days. Limp and eye complications can also be seen in about 5 to 10 percent of infected animals. Agalactiae may also be found in respiratory tissues.

Mycoplasma Agalactiae Diagnosis and Differentiation

In an experiment carried out in Jordan in 2003, out of 62 collected milk samples and 310 nasal mucus samples, the bacteria could be differentiated, respectively, in 17 and 12 cases that it was as 8 of 62 and 7 of 310 samples for goats. Three important species of Mycoplasmas were reported in this study, most of which usually seen in goats, and the unexpected finding was Mycroplasma putrefaciens, because the most frequent bacteria that are usually differentiated in this disease are agalactiae (Wu and Kado, 2004).

Experiments Conducted in Iran

Entessar and Borry for the first time reported the existence of agalactiae in Iran in 1963 (Arabi and Vend 2011; Borry and Entessar, 1963). Aerabi and Sotoudenia reported 23 cases of agalactiae in different regions of Iran. According to these studies, 490 samples of the milk of sheep and goats were collected for agalactiae differentiation, among which 96 cases of the disease were identified by biochemical tests and 23 of them were finally verified by serology tests (Arabi and Soyoodehnia, 1984; Arabi and Naser, 2010).

MATERIALS AND METHODS

Culture Mediums Include:

1. pplo broth
2. pplo agar

We pour the milk samples, under sterile conditions and near the flame, into some test tubes containing pplo broth, hold their tops on the flame and immediately put their covers on. Afterwards, tubes are stored in an incubator at 37 centigrade degrees, 70% humidity and 6% carbon dioxide. After 48 hours of incubation they are subcultured in a new pplo broth medium. For this purpose, 1 to 2 ml pplo broth is transferred to the new mediums. 14 to 28 days after the first and second cultures, cultivation continues in a solid medium and after some time samples are checked (Murray *et al.*, 2003).

DNA Extraction from Milk Samples

1. At first, milk samples were moved from the freezer to the refrigerator to let their ice melt down. Then, the samples were centrifuged in a refrigerated centrifuge for 15 minutes at a speed of 13000 rpm, at 4° centigrade.
2. The upper liquid was carefully separated with a sampler, while near the flame, and 1 ml of lysis buffer was added and properly mixed with the remaining sediment.
3. The samples were stored in the incubator for 24 hours.
4. The samples were put inside the benmary with a temperature of 85° c for 1 hour and were shook every 10 to 15 minutes.
5. An amount of 500 λ of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) was added to the samples.
6. The samples were placed in an ice container for 10 to 25 minutes.
7. The samples were centrifuged at 13000 rpm again, for 10 minutes this time. After the centrifugation, 3 phases were formed in the samples, the uppermost of which was carefully separated with a sampler and moved to another sterile microtube.
8. 500λ of Chloroform was added to the upper layer that was separated in the previous step.
9. The samples were centrifuged another time (13000 rpm, 10 min).
10. The upper liquid was separated, moved to another sterile microtube and added 1 ml pure ethanol.

11. The samples were incubated at a temperature of -20°C for 24 hours.

12. The samples were removed from the temperature of -20°C and were put at room temperature so that their ice melted down.

13. The samples were centrifuged at 4°C again (13000 rpm, 10 min).

14. The upper liquid was thrown away and 1 ml of 70-percent alcohol was added to the remaining sediment.

15. After the centrifugation of samples, the upper liquid was thrown away.

16. The covers of microtubes were removed so that the alcohol could blow out.

17. 20 µl of TE Buffer was added.

18. The samples were put in the benmary for 1 hour at 65°C so that DNA dissolved in them.

19. Samples were stored at a temperature of -20°C until the time of PCR test.

PCR Set-up

The DNA samples extracted from bacteria were put in the benmary at 65°C for 15 minutes and then were short-centrifuged. Following primers were used:

FS1: 5′-AAAGGTGCTTGAGAAATGGC-3′

FS2: 5′-GTTGCAGAAGAAAGTCCAATCA-3′

PCR in Milk Samples

The extracted DNA from the milk samples was first placed in the benmary at 65ºC for 15 minutes and after a short centrifugation and the addition of ingredients required for PCR with the following measures, was placed in the PCR.

H2o=27µl	10Xbuffer=5µl	F primer=4µl	R primer=4 µl	dNTP=2 µl
MgCl2=1.5 µl	DNA=5 µl	Tag DNA	polymerase=1 µl	Total=50 µl

Afterwards, it was programmed like the following:

Denaturation	94o	1min
Annealing	60o	1min
Extension	72	1min
Final Extension	72o	10min

RESULTS AND DISCUSSION

Results

The results of the experiments carried out on the 367 milk samples collected from sheep and goats are as follows. Of 367 cultured milk samples, 20 samples (5.4%) were reported positive (diagram 1).

These samples were evaluated according to the colonies grown on solid medium. The growth of Mycoplasma colonies makes the culture medium opaque (on the left of the image).

The colonies grown on the sold medium that looks like scrambled eggs.

The results of PCR for 20 samples of grown Mycroplasmas on a pplo agar medium with FS-1 and FS-2 primers of Mycoplasma agalactiae showed that of the foregoing 20 samples, 5 cases (25%) were Mycoplasma agalactiae.

The result of PCR on 367 milk samples, in 11 cases (2.9%) with Mycoplasma agalactiae primers came out positive.

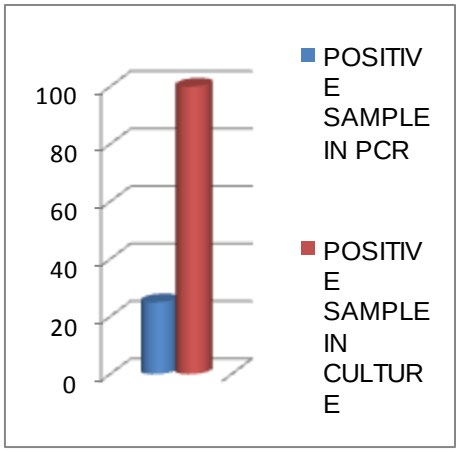

Diagram 1: The results of PCR from the positive culture

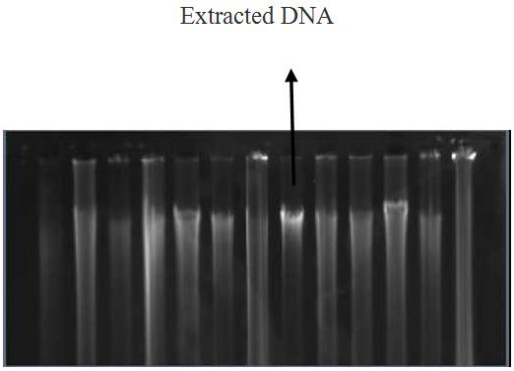

Figure 1: Extracted DNA of Mycoplasma agalactiae

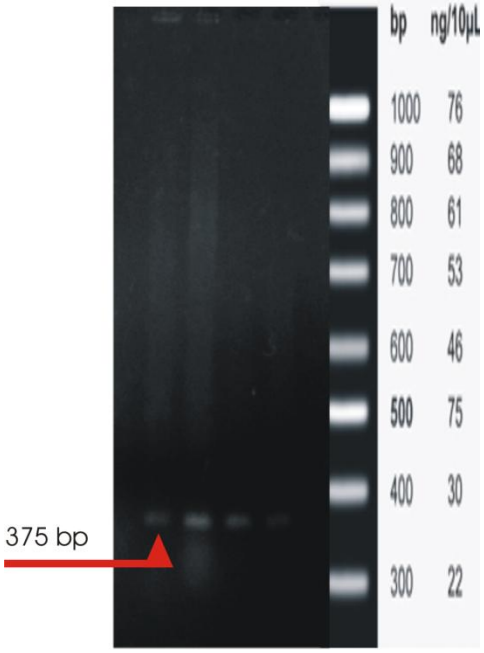

Figure 2: The result of Mycoplasma agalactiae PCR from the milk samples that indicates the point 375 bp

Discussion

Egwu and colleagues separated Mycoplasma agalactiae from milk samples using the method of culturing in pplo medium in 1999. In this series of studies done in Nigeria, 24 milk samples were collected from goats with mastitis signs and 28 samples were collected from apparently healthy goats. The results of these studies indicated that Mycoplasma agalactiae existed in 39.1% of goats with mastitis and 42.9% of them that were apparently healthy (Egwu *et al.*, 1999). Pirali and colleagues studied on Mycoplasma agalactiae in the sheep infected with agalactiae disease in western Iran in 2007 through which 20 cases were reported opposite after PCR test. 77 percent of the sheep were totally infected in this region. Primers mgpo and mgbo were used in the PCR test. Out of 47 tested milk samples taken from pregnant goats 44 cases had a positive PCR result and different results were derived from other tested samples. These results demonstrated that 20 percent of this region's sheep were infected with Mycoplasma agalactiae, being the cause of infectious agalactiae and actually reported for the first time in Kohgiluyeh and Boyer-Ahmad Province (Piral and Ebrahimi, 2007).

Subramaniam and colleagues studied some specific properties of Mycoplasma agalactiae in 1998 and determined the sequence of gene uvrC, which is a DNA fixer, in Mycoplasma agalactiae and Mycoplasma bovis by using PCR. This sequence was used in order to design primers for Mycoplasma agalactiae and Mycoplasma (Subramaniam *et al.*, 2008).

Bandeiral and colleagues attempted to identify Mycoplasma agalactiae by PCR method in Brazil in 2008. Their goal was to verify the existence of Mycoplasma agalactiae in the goats that had been made free of these bacteria before (Bandeira1 *et al.*, 2008). A total of 120 milk samples were taken and buffered glycerol saline was used for their preservation. Out of those 120 samples 9 cases (7.5%) of positive PCR were reported and a 360 bp DNA fragment was obtained.

In the current study, 367 milk samples from the goats and sheep with clinical signs of this disease were analyzed that resulted in 11 positive cases (2.9%). Working on separated samples, out of 20 samples having used primers FS1 and FS2 (375bp), 5 cases of Mycoplasma agalactiae were obtained.

Suggestions

As this disease causes economically considerable damages to herds, relying on a faster method of discovery will certainly be of high importance in prevention and treatment processes. By means of PCR method, the cause of disease can be detected in 5 hours that is more valuable compared with culturing method that takes days. Therefore, early and fast diagnosis and quarantining infected herds and seemingly healthy carriers from the healthy can highly prevent the disease from infection to others. On the other hand, due to higher accuracy and sensitivity in comparison to other methods, this one also lowers the probability of diagnostic flaws. However, culture method is also recommended to be used in parallel as a very-good-working gold standard.

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