

## Full Length Research Paper

# Application of PCR for diagnosis of contagious Agalactia in Khuzestan Province- Iran

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DNA amplification technique which is called PCR is a precise and specific test method and nowadays is also used for identification of *Mycoplasma*, the causative agent of contagious agalactia in sheep and goats. It also avoids the antigenic cross reaction and variability that hamper serological methods. Several sets of primers are available for different members of *Mycoplasma* such as *Mycoplasma agalactia*, *Mycoplasma capricolum*, *Mycoplasma mycoides*, and *Mycoplasma putrefaciens*, which cause the enzootic infections frequently, in some area of Iran, even in Ahvaz, the central city of Khuzestan province. Several methods for the detection of *Mycoplasma* such as culturing and serological tests have been published. These tests would require by making the culture media (agar and liquid media), time consuming about (3 to 5 weeks to grow detectable colony), and even some *Mycoplasma* species were difficult to detect with these methods. A specific PCR assay was developed as a diagnostic test, for detection of *Mycoplasma* infections, carried out for evaluating 47 samples of synovial fluid, milk and eye swabs from sheep's and goats which about 19.1% of them were showed positive for *M. agalactia*. According to our results only few samples grew in culture, (8.51%). Use of PCR test with high specificity and sensitivity for detection of *Mycoplasma* has become increasingly widespread, therefore for a quick detection of infection caused by *M. agalactia*, PCR test application is recommended.

**Key words:** Sheep, goat, milk, *Mycoplasma*, contagious agalactia.

## INTRODUCTION

There is six of *Mycoplasma* in a group which cause contagious agalactia in sheep and goats that has been known for about two centuries. *Mycoplasma* belong to a group of bacteria, without cell wall while possess a cytoplasmic membrane. They are also found as free living organisms. *Mycoplasma* contains a small gene and similar to viruses which is able to pass through the filtration. They are between bacteria and rickettsia organisms from systematic point of view, so the organisms can be divided by binary fission and they are gram negative.

The clinical disease was 1st described by Metaxa in

Italy 1816, and the contagious was named by Brusasco in 1871, Madanat et al. (2001). The contagious agalactia is one of the major infections of goats and sheep which are caused by *Mycoplasma agalactia*. The syndromes such as mastitis, arthritis, conjunctivitis and sometimes pneumonia may observe with the other *Mycoplasmas* such as *Mycoplasma mycoides* subsp, mycoides large colony (LC), *Mycoplasma capricolum* subsp, capricolum and *Mycoplasma putrefaciens*.

The contagious agalactia are reported from Mediterranean area, Balkan, south west Asia countries like Turkey, Iran, Iraq , center east of Africa, and united state of America in endemic form. It is very difficult to control its transmission from diseased to healthy ruminant, because of insanitation and a poor hygienic production of sheep and goat (Tola et al., 1997). The agalactia infection can occur in a single or sometimes in a group of ruminant in a herd. Though such infections are

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able to cause the economical loss, for example; lactating animals are usually manifested by mastitis, along with decreasing of milk production or No milk at all. The young animals are most commonly infected because of their sensitivity to the disease and may die.

The morbidity of infection is about 30 to 60% in mature animals, while the mortality in young animals is 40 to 70%. On the other hand goats are more susceptible than sheep and their symptoms include bacteremia accompanied by fever, sometimes abortion may occur in pregnant animals, which is due to inflammation of uterus (Bergonier et al., 1997). In addition, the male animals show inflammatory changes in their reproductive organs (testes). So the disease in a herd can persist for several months. The contagious agalactia is reported from several parts of Iran provinces with common outbreaks are as follows, at west north of Azerbaijan, Zanjan, Kermanshah, Lorestan, south and west Fars and Khuzestan, even in central parts, Esfahan, Yazd, Semnan and at north east Khorasan.

More than 200 species of *Mycoplasma* which causes infection in human, plants and animals, are recognized up to now, of which about 40 species are caused disease in ruminants and some domestic animals. From economical point of view there are three kinds of *Mycoplasma* infections that are very important according to OIE reports which have been listed as follows.

- 1- Contagious bovine pleuropneumonia, which is caused by *Mycoplasma mycoides* subsp *mycoides*.
- 2- Contagious caprine pleuropneumonia in goat by *Mycoplasma capricolum* subsp *capricolum*.
- 3-The contagious agalactia that is the main causative agents of agalactia outbreaks in the world.

During an outbreak of mycoplasmosis in a field, the disease can be detected primarily from the clinical signs. While in a single animal it is a little bit difficult to detect the disease, but for exact diagnosis of the disease it may be possible by sampling and culturing of milk, eye swab and joint secretion, even it is possible to diagnosis the carrier one. In case of growth of organisms in culture media, several tests such as serological like hemagglutination inhibition (HI), indirect immunofluorescence test, complement fixation test (CFT) and ELISA should be performed to get identification of *Mycoplasma* species. Ceccoralli and Funtanelli (1950) mentioned that by the help of CFT test agalactia could be recognized, but with exact studying the test, they found that it will be useful for detection of disease in a field, but it is not trustable because the results of CFT test may show false negative reaction (Ghader et al., 1996).

So for diagnosis of agalactia in a herd, the above tests are not very sensitive, on the other hand the serologic and bacteriologic methods may be useful to detect the species of *Mycoplasma*, while antibody titrations is very important to diagnose the diseases in the field. In most

of the cases because of the negative results of serological test and the rapid spread of *Mycoplasma* in a herd, a quick, sensitive and specific method is required for detection of *Mycoplasma* infections. Therefore use of DNA probe has been reported for quick detection of *Mycoplasma* agalactia infections such as, *M. capricolum* and *M. bovis*.

According to study of Tola et al. (1997) and Dedieu et al. (1995) polymerase chain reaction (PCR) method on the base of DNA extraction has been described to detect *Mycoplasma*. It is a sensitive, quick and specific test, Hopert et al. (1993). In this enzymatic reaction a specific segment of DNA from a complex of nucleotide can be diagnosed. Some times in a clinical sample, there may be more than one species of *Mycoplasma*, for this purpose before the biochemical and serological test being performed the colony of *Mycoplasma* should be purified and cloned.

To work and perform culturing, biochemical and serological tests would take long times at least two weeks while by PCR due to no need of purification and cloning, pathogenic *Mycoplasma* are easy to detect even within the mixed organisms in a sample, without spending long times. It requires at least 2 to 3 h (Lorusso et al., 2007). Several different methods of PCR have been described and used on the bases of different gene by researchers, (Chazel et al., 2010; Ayling et al., 1997). In this manner PCR is a more sensitive and precise detection method than bacteriological growth method.

## MATERIALS AND METHODS

### Collection of samples

A total of 47 samples were randomly collected from milk, eye swab and synovial fluid (with the help of disposable syringes) from goats and sheep's of the rural areas such as Al-Baji, Borvayeh and Sajadieh around the Ahvaz city. The samples were immediately placed in test tubes containing *Mycoplasma* transport medium (made by Hesarak, Razi Vaccine and Serum Research Institute of Iran), and they were kept beside ice until transferred to the laboratory for PCR test. Before taking the milk samples first the teat was disinfected by 70% alcohol and after flow up 2 to 3 drops of milk, some drops were taken in a tube which contains *Mycoplasma* broth medium.

### The PCR test

#### DNA extraction

Because the *Mycoplasma* lacking cell wall, the DNA extraction was done by a simple boiling method, as per Cinagen supplier catalog, No: DN8115C. Briefly at first one ml of each sample that was collected and incubated at 37°C for 24 h for isolation of *Mycoplasma*, was transferred in to 1.5 ml micro tube and kept at room temperature for 10 min. After Centrifugation at 13000 rpm for 15 min the supernatant was discarded and to the sediment was added 100 µl proteinase enzyme and after covering the leads of micro tube with Para film, boiled for 10 min in a beaker containing boiling water, then after addition of 400 µl of the lyses buffer and

subsequently 300 µl of precipitation buffer were added and shaken by vortexing, then was frizzed at -20°C for 20 min. Again Centrifugation step was repeated for 10 min as above. Then supernatant was discarded and 1 ml wash buffer was added to each micro tube, and centrifugations were done for 5 min same as above. Discard supernatant, and dry the micro tube with a tissue paper. Then add 50µl solvent buffer to each micro tube and heat at 56°C for 15 min, afterwards it was incubated on ice (0°C to cause thermal shock) for 1 min, and then Centrifugation at 13000 rpm for two min. Now keep in a refrigerator till the PCR test being down.

## Preparation of PCR mixture

### Preparation of PCR master mixture for 10 samples

Add 25 µl PCR buffer, 5 µl DNTP (0.1 to 0.2 m/ M), 3 µl MgCl<sub>2</sub>, 11 µl Primer I and 6 µl primer II.

P1- 5'- AAAG-GTGCTTGAGAAATGGC-3'

P2- 5'- GTTGCAGCA GAAGAAAGTCCAATCA-3'

Add 2.5 µl Tag DNA polymérase (Pharmacia Biotech Uppsala)

By addition of 207.5 µl D/W, make total volume 260 µl and the mixture is mixed well by vortexing.

### Distribution of prepared master mixture in 0.5 ml, micro tubes

3-1- for each sample 20 µl of master mixture was aliquated in a 0.5 ml micro tube and 5 µl of extracted DNA was added to get a total volume 25 µl.

3-2- 20 µl of master PCR mixture and 5 µl of distilled water were used as a negative control.

3-3- 20 µl of master mixture with 5 µl of known positive sample in a micro tube was used as a positive control.

3-4- add a drop of mineral oil to each tube.

## Thermo cycling process

All PCR reactions were processed in a Perkin Elmer DNA thermo cycler under an optimized (MAG) program which consisted of primary denaturizing at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, 1 min at 65°C for denaturizing, annealing, and extension phases respectively. The process was followed by an additional period for the final extension at 67°C for 10 min.

## Electrophoresis process

Twenty microtelrel of amplified mixture (PCR product) of each micro tube were run on a 1.5% agarose gel and subjected to electrophoresis for 2 h at 110, V. After staining with 0.5 µg /ml of ethidium bromide, amplified bands were visualized and photographed under UV transilluminator. The fragment size of 1.7 kb was considered as specific for *Mycoplasma agalactia*. Figure 1

## RESULTS AND DISCUSSION

Forty seven samples included (milk samples, eye swabs and synovial fluid) were examined by PCR method, six samples from the Al-Baoji village and three samples from Sajadieh area were detected as positive for *Mycoplasma agalactia* and the rest of the samples were negative. So

with respect to number of the samples and the results obtained, the rate of agalactia infection was about 19.1%, which show presence of the causative agents of agalactia in herds of Ahvaz and its rural area. Setoodenia and Aarabi, (1993) reported that out of 28 samples 10.7% were shown positive in Khuzestan province for *Mycoplasma agalactia* (Table 1). In the present study the prevalence of agalactia infection in Ahvaz and their rural area is confirmed. Of course the chance of surveillance of *Mycoplasma* and its persistence in warm and desert environment is very less and is unable to survive out or in a herd. But in a very rare condition such as frizzing and other status, like bacteria in animals body (carrier), *Mycoplasma* could be able to survive with preserving its virulence factors, for a long times. Therefore the only way for infection to spread out is by body secretions like milk, sneeze and cough.

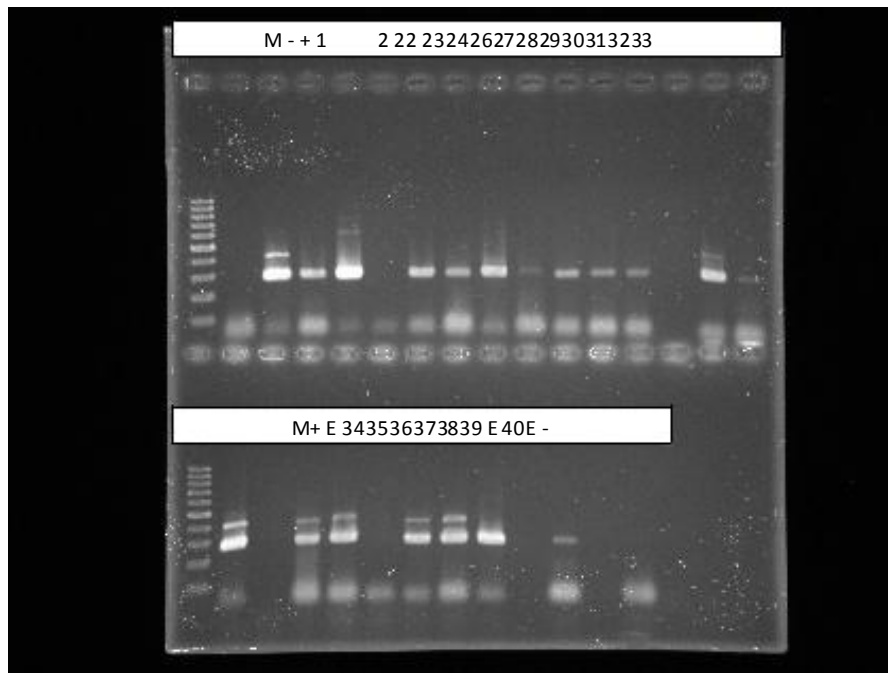
An experimental study of *Mycoplasma* infection showed that the infectious agents can alive at least for 56 days in the lymph node of the neck and spread slowly within the herd. While during other infections organisms persist lesser than them, (Sanchis et al., 1998). Meanwhile after occurrence of an agalactia infection on 200 animals of a herd, gradually the cure will happen among the animals of that herd, but sometimes *Mycoplasma agalactia* may be isolated even after three months of its clinical signs being recorded (Rodrigues, 1996). So in such condition because of no quarantines and poor sanitations, the infecting agent will survive and even by suitable weather and wet condition it goes to increase. By this why these agents cause an outbreak from an area to other places of the world, and have been reported from all countries of the world except Britain.

Primary detection of contagious agalactia is on the basis of epidemiological information and its clinical signs in specific area that include mastitis along with loss of milk, keratoconjunctivitis and arthritis. The disease in some endemic area with a mild status shows the similar clinical sign that is mastitis without keratoconjunctivitis and arthritis. In the mastitis form, the disease occurs immediately after delivery. The routine method of diagnosis of *Mycoplasma* infection is on the base of ordinary classical methods such as serological, (immune fluorescence), and biochemical tests for *Mycoplasma* identification which are time consuming and the results obtained are difficult to interpret. So a quick and specific test is very useful to detect *Mycoplasma* disease in a herd. Nowadays it seems that detection of *Mycoplasma* by molecular technique is more reliable than the clinical methods as molecular method of detection is upon DNA genome and its sequences and short time of diagnosis.

In the present study the identification of isolated *Mycoplasma* in Iran from the molecular genome point of view is upon the base of the 40 KDa membrane protein of *Mycoplasma*, which is an immunogenic and one of the main virulent factors of bacteria as well as being very specific for *Mycoplasma* (Mahdavi et al., 2009). Tola et al. (1997) have planned two primers for detection of

**Table 1.** Shows samples distribution by area and type as well as PCR and culture positivity rate.

Area	Milk	Eye swabs	Synovial fluid	PCR + ve samples	PCR %+ ve	culture + ve	Culture %+ ve
Al-Baji	18	5	1	6	12.7	3	6.38
Borvayeh	10	-	-	-	-	-	-
Sajadieh	12	1	-	3	6.4	1	2.12
Total	40	6	1	9	19.1	4	8.50



**Figure 1.** Agarose gel electrophoresis of PCR product for illustration of *Mycoplasma agalactia* as compared with positive control (+). No, 32, 34, 35, 37 and, 38 are *M. agalactia* positive, while the rests of them are showing with a single band are only genus of *Mycoplasma*. E=empty.

*Mycoplasma* by use of agarose gel electrophoresis, and southern blot analysis. The primers' were replicated for DNA of *Mycoplasma agalactia* while the other *Mycoplasma* with the rest of the other bacteria will not replicate able. Thus the test confirmed that PCR is a precise and a quick method for detection of *Mycoplasma agalactia* disease (Dedieu et al., 1995).

Hotzel et al. (1995) tried to advance the detection of *Mycoplasma agalactia* with a set of primers for *Mycoplasma*. He reached the results that PCR is faster than the traditional tests and it can be used as confirmatory test in the diagnosis of Mycoplasmosis, even after storage of the samples at -20°C for 24 months.

According to the researcher's reports on a variety of samples the most positive results obtained, are related to eye swabs. While others believe that the similar positive results are obtainable with milk. For identification of *Mycoplasma* can also use other samples such as lymph-

node, and blood (in septicemia cases), although preparation of these samples have its own difficulties, for example in the case of blood, only in acute form of septicemia, blood sample can show positive but on the late phase of the disease never get positive for *Mycoplasma*. There are some reports about use of ear swabs, but due to possible presence of ear mite, it is not a suitable sample for *Mycoplasma* infection. Though, there are some rare positive results by such samples. The mites may become as a carrier for transmission of *Mycoplasma* in a herd. It is impossible to isolate *Mycoplasma* from canal of ear in healthy but not diseased animals (Zendulkova et al., 2007).

## RECOMMENDATIONS

As mentioned, by comparing the results of serologic tests

like CFT, ELISA, bacteriological and biochemical methods, with that of PCR, PCR with high sensitivity and specificity is more useful. On the other hand, to prevent spreading of agalactia in herds, at borders of a country during animal's transportation in or out, the PCR tests are recommended to find out the carriers.

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