

Full Article

Detection of *Mycobacterium avium subsp. paratuberculosis* in Cow Milk Using Culture and PCR methods

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Received 20 Jun 2011; accepted 5 Sep 2011

ABSTRACT

Mycobacterium avium subspecies paratuberculosis (MAP) is the cause of John's disease also called paratuberculosis. This is economically one of the important infectious diseases in cattle and ruminant husbandry. This disease is manifested as granulomatous enterocolitis, lymphadenitis and inflammation local lymphatic vessels. The typical sign of this disease is progressive loss of weight. Considering the importance of detection of this disease in this study, two methods, culture and PCR, were used for the identification of this microorganism. In this study 100 milk samples from apparently healthy cows and 100 milk samples from cows that have been suspicious of John's disease were taken from in Sarab, East Azarbaijan, Iran. Direct microscope observation after ziehl-neelsen staining was done. Then, bacterial culture on specific medium was carried out, and finally, identification of *Mycobacterium avium subsp. paratuberculosis* was examined using PCR and specific primers. Using direct observation, culture and PCR analyses showed that from 100 healthy cow milk samples, 8, 9 and 12 samples were positive MAP for each method respectively. The results of direct observation, culture and PCR analysis on affected cows were 15, 40 and 44, respectively. The results of this study showed that culture and PCR analyses methods are important in the identification of the causes of this disease. Therefore, considering the frequency of the disease in the studied region, either of those methods can be used in the microorganism identification.

Keywords: John's disease, Milk, Culture, *Mycobacterium avium subsp. paratuberculosis*, PCR

INTRODUCTION*

The John's disease was recognized for the first time in 1895 by Johne and Frothingham in a cow infected by

chronic swollen intestine with thickening and wrinkling of intestine mucus along with presence of Acid-fast bacilli. In 1906 Bang discovered that this disease was not tuberculosis, so named it John's. At the first stage, cause of disease was called *Mycobacterium johnei* then it changed to *Mycobacterium paratuberculosis*, but

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now it is known as *Mycobacterium avium* subspecies *paratuberculosis* (Anzabi et al 2005).

John's disease has spread universally and has ever-increasing prevalence in all countries. Human being could be affected through raw milk or pasteurized milk and also meat and environment as secondary sources exposed to *Mycobacterium paratuberculosis* (Donaghy et al 2008, Tabatabaïi & firoozi 2001). This disease has been observed in cow, to some extent in sheep and goat. The highest rate of affliction is usually under the age of 30 day, but this disease with clinical symptoms hardly happen between the ages of 2 to 5 years old. In intensive infections, however, even one year old calves show symptoms of disease.

Because of slow pace of the disease prevalence John's disease occurs individually (Tabatabaïi & firoozi 2001). The studies show that in the cattle addicted to John's disease through different ways (clinical case, histopathology and cultivation), the sub clinical cases go further than clinical cases, and such domesticated animal will be the main factor of spreading contamination among the cattle. This problem will become even more complicated when contaminated milks to be used for feeding calves. The stool of the afflicted animal could be the main source of this infection. The disease starts with touching of disposed animal and its contact with contaminated stool, and also having contaminated food and drink in contact with stool.

The length of incubation period (2 years or more) will cause repelling of bacteria from contaminated animal to start 18 months prior to the appearance of evident symptoms (Frank 1994). Contamination of breast in contact with stool and presence of disease factor in colostrums or milk may cause the ingestion of bacteria by calf. Breast infusion in cow created by bacteria leads to limited topical spread, but of the breast does not happen. Bacteria are isolated from cow's womb: however, disease symptoms have not yet been observed in the infected fetus. Bacteria has also been isolated from sperm and genital organ of infected animal, and because of resistance against freezing conditions and

antibiotics added to the sperm, internal infection of womb is caused (Frank 1994).

Some researchers believe many factors such as: concentrated husbandry system, acidic soil, insufficient feeding, transport stress, feeding time, accouchement, and diarrhea caused by virus are effective in this disease (Doyle 1998). This bacterium is capable of remaining in the field for a long time without multiplication, and such field remains infectious for one year. Alkaline soil is very effective in the presence of clinical symptoms. Other effective factors influential in animal affliction consist of age, stress, infection dose, and weakening factors of small intestine mucus sectional lymphatic gland, tonsils (to some extent) and lymphatic glands beyond pharynx (Cetinkaya et al 1996, Whittington et al 2000).

According to survey by animal husbandry organization, existence of John's disease has been proved in the states of Iran (Tabatabaïi & firoozi 2001), East and West Azerbaijan, Lorestan, Khuzestan, Semnan, Tehran, Fars, Hamadan, Khorasan and Kermanshah (Tabatabaïi & firoozi 2001). However, there is not enough data showing the incidence and prevalence of the disease among cow, sheep and goat populations of different regions in Iran (Anzabi et al 2005). Albeit in different reports higher percentage of prevalence has been reported among Milking farms in comparison with the Feed-lot farms. For the first time the John's disease was recognized in Iran by Khalili and Talachian in 1960-1961. The disease agent was isolated from stool of Jersey cows imported by Abadan Iranian National co. and reported to be the infection factor of imported cows (Tabatabaïi & firoozi 2001). In 1972 the afore-mentioned persons investigated the epidemiology, clinical and pathological aspects of John's disease on sheep and goats (Anzabi et al 2005). Diagnosis of this disease carried out through biopsy sampling, stool cultivation, direct survey by microscope, tracking DNA and serology testing, among which cultivation test and molecular diagnosis play important roles. Therefore in the survey of John's

disease milk samples of both infected cows and healthy cows were examined using both methods.

MATERIALS AND METHODS

Milk sample culturing. Milk samples were prepared in the sterile containers of 50ml and centrifuged in 3000g for 15 minutes. Then 5ml of the produced cream was transferred to another sterile test tube. After removing the upper part of residue, disinfection was done both on the residue and the cream by using 0.75% solution of Hexa duodecil Peridinium Chloride (HPC Sigma) For this purpose 20ml of this material (almost 4 times of residue or cream) was added to the tube, containing residue and cream, and was kept in room-temperature for five hours after mixing. Then the contents of tubes centrifuged in 3000g rpm for 15 minutes and upper part of liquid were removed. Afterwards the pH of residue was adjusted to 7.2-7.4 and transferred to the 4 Herrold' egg yolk medium (which 3 of the media containing minimum 2mg/1000ml Mycobactin or three times of *Mycobacterium fleii* extract, and one medium without Mycobactin).

Preparing specific medium for separation of John's factor or Herrold' egg yolk medium. Specific medium for separation of John's factor Agar 15.3g, Pyruvate sodium 4.1g, Glycerol 27ml, Meat juice 2.7g, Sodium chloride 4.5g, Peptone 9g, Distilled water 870ml were prepared and pH medium was adjusted between 7-7.5. Next, 2mg of Mycobactin was dissolved in 4ml of ethylic alcohol and added to the solution. After autoclave of said 6 sterile eggs were added. At the end 5.1ml malachite green 2% was added to the solution. It should be mentioned that preparation of Herrold medium follows the same procedure without adding Mycobactin.

Extraction of DNA genomic mycobacterium by using cetyl-tri-methyl-ammonium-bromide (CTAB). The method was carried out by Van Soelingen and his colleagues stated in 1991 which is a combination of

chloroform/isoamino alcohol and isopropanol and used for extraction and DNA (11).

PCR. 3µl of extracted DNA was added to PCR master mix with final volume of 20µl each vial containing 1.5 µl, MgCl₂ (from 50mM, 2µl buffer 10x, 2µl of dNTP (from 10m mole stock), 1µl from each primer (from mix primer 10 pM, 1µl Taq polymerase enzyme (Cinnagen) 10µl water. PCR with thermal condition preliminary denaturation 94 °C/1min, followed by 35 repeated cycle 94 °C/1min, 59°C/45s, 72°C/1min and final cycle 72°C/10min was used, and the results by using electrophoresis in Agarose gel 1% were investigated. Standard strain of *Mycobacterium avium subsp. paratuberculosis* ATCC 19698 was used for control.

Method of testing. In this research microscopic direct test by doing specific staining of ziehl-neelsen also specific bacterial culturing on milk samples of 100 healthy-looking cows and 100 cows suspected of MAP infection related to the number of cow-keeping in the region of Sarab which used to have background of this disease was carried out as follows:

With reference to the selected cow fields the milk samples in the sterile containers of 50ml and observance all hygienic principles were prepared and under sterilized condition inside packs of ice was transferred to the laboratory. The mentioned samples were centrifuged in 3000g for 15 min and cream part of each sample was removed. Then under sterilized conditions 5ml cream of each sample was selected and transferred to sterile tube test and after removing the upper part liquid of same sample 5ml of remaining residue (sediment) in the bottom of said tube it was transferred to another sterilized test tube. Then stage of removing contamination either on residue or cream of the samples by using 0.75% solution of HPC was carried out.

Finally the disinfected residue and cream related to each group were separately transferred 4 specific cultivation of Herrold' egg yolk. It should be noted that in each quadruplicate series 3 medium containing Maycobactin and another was without it.

After cultivation (culturing) all media were kept in incubator of 37 °C for 4-12 weeks. Simultaneous with cultivation of each sample, immediately after disinfection stage in exchange for each sample, at least 3 cultures were prepared and investigated for the presence of *Mycobacterium avium subsp. paratuberculosis*.

RESULTS

After the direct test and culture on the samples, PCR through using specific primers showed in Table 1 was carried out. Electrophoresis was done for PCR products and as was expected, bands with 402bp size were observed.

Table 1. Characterization of primers used in PCR method.

Nucleotide sequence	Fragment length	Primer name(10)
5'-CAGTCACCCCGCGCCGGTA-3'		(F)locus 225
5'-TCTACTGACCCGCAGATCGAA-3'	402bp	(R)locus 225

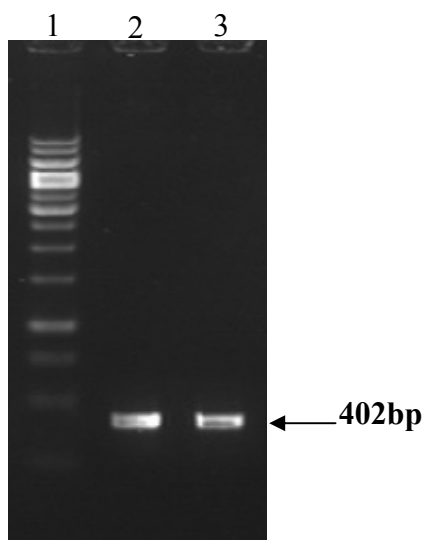


Figure 1. Electrophoresis model of PCR product on agarose gel. Lane 1. DNA marker (1kb), Lane 2. Positive control sample ATCC 19698, Lane 3. Clinical positive sample

Table 2 shows direct test, culture and PCR results as seen in this table in comparison the number positive samples by PCR method was higher than direct test and culture.

Table 2. Number of positive samples detected by different methods and P-Value.

sample	Milk	Direct test	Culture	PCR	P-Value
Healthy cows (n=100)		8 (8%)	9 (9%)	12 (12%)	0.609
Affected cows (n=100)		15 (15%)	40 (40%)	44 (44%)	<0.001

DISCUSSION

The study in the recent years regarding American cattle of dairy cattle indicated that nearly 40% of these cows were infected with agent of John's disease and this led to a great economical loss in the dairy products industry (Lisle GWY & Collins 1993).

With regard to the subject of repelling MAP what hygienic importance may have for domesticated animals and human beings there are numerous articles, so that several studies in the USA concerning John's disease indicated that the cows with chronic infections and had positive response against stool cultivation in comparison with cattle having negative response, produced 15% less milk. The similar findings were reported through ELISA test, but the results show harmful increase in milk production of cows (Bono et al 1995, Dwiredi & Sarin 2000).

With regard to importance of duration of MAP in raw and pasteurized milk which is important not only from the hygienic point of view and feeding animals especially new born calves, but also human public health concerning consumption of milk and dairy products. In the experiments carried out in Britain may be one of them is more important and interesting. The experimental research in Britain proved that the MAP bacteria present in the natural milk of cows infected to John's infection in comparison to the cultivated strains

of same bacteria in the laboratory which added to the test tubes containing milk, were more resistant against heat (Lisle GWY, & Collins 1993). Which this case could explain the difference in the experiments over natural contaminated milks and milks that contaminated to MAP bacteria experimentally. Sometimes the results are not similar and do not meet to our expectations (Lisle GWY, & Collins 1993, Moreira *et al* 1999, Nordlund *et al* 1996).

Of course there are public criticism against laboratory findings with regard to duration of bearing pasteurization heat by John's disease factor, since through creation of similar laboratory conditions and pasteurized milk carrying industrial scale through HTST (High Temperature Short Time) method by continuous heating, it's possible to make milks with natural contamination (Chiodini *et al* 1993, Lisle GWY, & Collins 1993, Pavlik *et al* 1999, Sanderson *et al* 1992).

Tohidi moghadam *et al.* conducted a study on cow milk and fecal samples for detection of *Mycobacterium avium subsp. Paratuberculosis* using PCR method, the result there study indicated that 41.1 (27.3%) samples of milk was indicated as positive using PCR. The result of this study was inline with the finding of Tohidi moghadam *et al.* (Tohidi moghadam *et al* 2010).

With regard to the finding of this study, and the comparison of these three methods in diagnosing this infection disease, it seems that molecular experiments, if available, can prove highly as valuable as culture in diagnosing infection disease.

There was not a significant relationship between the kind of methodology (diagnosis) used and the test result in healthy cows ($P=0.609$), while there was a significant relationship between the kind of diagnosis methodology and test result in the affected cows ($P<0.001$).

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