

## Comparison of Culture and PCR Methods for the Detection of John's Disease in Milk Samples

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**Abstract:** *Mycobacterium paratuberculosis* (MAP) is the cause of John's disease or paratuberculosis. This is economically one of the important infection diseases in cattle and ruminant industries. This disease is manifested as granulomatosis enterocolitis, lymphadenitis and inflammation local lymphatic vessels. The typical sign of this disease is the progressive loss of patient 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 cattle Sarab region. Direct microscope observation after ziehl-neelsen staining was done. Then, bacterial culture on specific medium was carried out and finally, identification of *Mycobacterium avium 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 analyses 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.

**Key words:** John's disease, milk, culture, *Mycobacterium avium paratuberculosis*, PCR, Iran

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### INTRODUCTION

The John's disease was recognized for the first time in 1895 by John 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 now it is known as *Mycobacterium avium* a sub species of *paratuberculosis* type (Anzabi and Tabatabai, 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 exposure to *Mycobacterium paratuberculosis* (Tabatabai and Firouzi, 2001; Grant *et al.*, 2002; Donaghy *et al.*, 2008). 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 happens between the ages of 2-5. In intensive infections however, even 1 year old calves show symptoms of disease.

Because of slow pace of the disease prevalence John's disease occurs individually (Tabatabai and Firouzi, 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 and Cook, 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 swelling the breast does not happen. Bacteria are separated from cow's womb: however, disease symptoms have not yet been

observed in the infected fetus. Bacteria has also been separated 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 and Cook, 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. This bacterium is capable of remaining in the field for a long time without multiplication and such field remains infectious for 1 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). *Mycobacterium paratuberculosis* is resistant against cell internal demolition and could stay alive inside macrophage for weeks and be protected against humeral factors.

Oral fecal transfer is the major way of disease transmission. In the advanced stages of disease, transfer of bacteria takes place from digestion unit to the breast and its transfer is probable through milk and colostrums. It is also possible through inhalation of dust caused by contaminated fertilizer. In this case it seems that the bacterium leaves lungs by coughing and following ingestion enters to digestion tract (Whittington *et al.*, 2000). The sperms of infected animals may contain John's bacillus so there is the possibility of disease transfer through mating or artificial fecundation (Whittington *et al.*, 2000). According to survey by animal husbandry organization, existence of John's disease has been proved in the following states (Tabatabai and Firouzi 2001), East and West Azerbaijan, Lorestan, Khuzestan, Semnan, Tehran, Fars, Hamadan, Khorasan and Kermanshah (Tabatabai and Firouzi, 2001). However, there is no precise statistic with regard to abundance of this disease among cows, sheep's and goats in different regions of the country (Anzabi and Tabatabai, 2005). Albeit in different reports higher percentage of prevalence has been reported among milks in comparison with the fleshy cows. For the first time the John's disease was recognized in Iran by Khalili and Talachian in (1339-1340). The disease cause organized from stool of Jersey cows imported by Abadan oil co. and reported to be the infection factor of imported cows (Tabatabai and Firouzi, 2001). In 1351 the afore-mentioned persons investigated the epidemiology, clinical and pathological aspects of John's disease on sheep and goats (Anzabi and Tabatabai, 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 50 mL and centrifuged in 3000 g for 15 min. Then 5 mL 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 Hexadecyl Peridinium Chloride (HPC). For this purpose 20 mL 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 5 h after mixing. Then the contents of tubes centrifuged in 3000 g rpm for 15 min and upper part of liquid were removed. Afterwards the PH of residue reached to 7.2-7.4 and transferred to the 4 Herrold's egg yolk mediums (which 3 of the said mediums containing minimum 2 mg/1000 mL Mycobactin or multiple times of *Mycobacterium fleii* extract and one medium without Mycobactin) (Chiodini and Hermon-Taylor, 1993; Cetinkaya *et al.*, 1996; Whittington *et al.*, 2000).

**Preparing specific medium for separation of John's factor or Herrold's egg yolk medium:** Specific medium for separation of John's factor Agar 15.3 g, Pyruvate sodium 4.1 g, Glycerol 27 mL, Meat juice 2.7 g, Sodium chloride 4.5 g, Peptone 9 g, Distilled water 870 mL were prepared and pH of medium was adjusted between 7-7.5. Next, 2 mg of Mycobactin was dissolved in 4 mL of ethylic alcohol and added to the solution. After autoclave of said solution 6 sterile egg were added. At the end 5.1 mL 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 Cetil-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 residue.

**PCR:** About 3  $\mu$ L of extracted DNA was added to PCR master mix with final volume of 20  $\mu$ L each vial containing 1.5  $\mu$ L,  $MgCl_2$  (from 50 mM stock), 2  $\mu$ L buffer 10 $\times$ , 2  $\mu$ L of dNTP (from 10 mM stock), 1  $\mu$ L from each primer

(from mix primer 10 pmol), 1  $\mu$ L Taq polymerase enzyme and 10  $\mu$ L water. PCR with thermal condition preliminary denaturation 94°C/1min followed by 35 repeated cycle 94°C/1 min, 59°C/45 sec, 72°C/1 min and final cycle 72°C/10 min was used and the results by using electrophoresis in Agarose gel 1% were investigated. Standard strain of *Mycobacterium avium* under (paratuberculosis ATCC 19698) type was used for control.

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

With reference to the selected cow-keeping fields the milk samples in the sterile containers of 50 mL and observance all hygienic principles were prepared and under sterilized condition beside packs of ice was transferred to the laboratory. The mentioned samples were centrifuged in 3000 g for 15 min and cream part of each sample was removed. Then under sterilized conditions 5 mL cream of each sample was selected and transferred to sterile tube test and after removing the upper part liquid of same sample 5 mL 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's egg yolk. It should be noted that in each quadruplicate series 3 medium containing Maycobactin and another was without it.

After cultivation (culturing) all medium 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 microbes cultures were prepared and investigated for the presence of *Mycobacterium avium paratuberculosis*.

## RESULTS AND DISCUSSION

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 402 bp size were observed (Fig. 1). Table 2 showed direct test, culture and PCR results as seen in this table in comparison

Table 1: Characterization of primers

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

Table 2: Results of direct test, culture and PCR in clinical samples

Milk sample	Direct test	Culture	PCR
Healthy cow	8	9	12
Affected cows	15	40	44

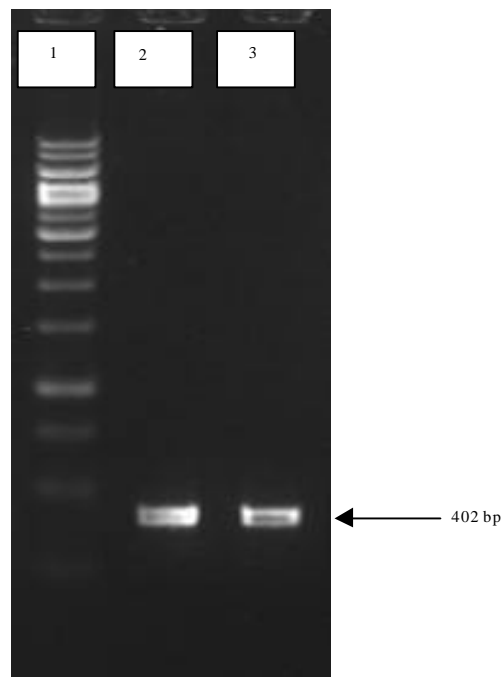


Fig. 1: Lane 1: DNA marker (1 kb), Lane 2: positive control sample ATCC 19698, Lane 3: Clinical positive sample

the number positive samples by PCR method was higher than direct test and culture. The study in the recent years regarding American cattle of milk-cows indicated that nearly 40% of these cows were infected with factors of John's disease and this led to a great economical loss in the dairy products industry (De Lisle *et al.*, 1993).

Numerous test regarding possibility of MAP bacteria presence in the commercial pasteurized milk in Britain as a part of broad investigation with regard to microbiologic quality of pasteurized milk and also row milk in this country indicated that at least 1.8% of the tested pasteurized milk samples considering the cultivation of said bacteria were positive. With regard to the subject of repelling MAP what hygienic importance may have for domesticated animals and human beings there are numerous studies so that several studies in the USA

concerning John's disease indicated that the cows having 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 (Carrigan and Seaman, 1990; Bono *et al.*, 1995; Dwivedi *et al.*, 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's 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 (De Lisle *et al.*, 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 the expectations (De Lisle *et al.*, 1993; Nordlund *et al.*, 1996; Moreira *et al.*, 1999). 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 method by continuous heating, it's possible to make milks with natural contamination (Sanderson *et al.*, 1992; Chiodini and Hermon-Taylor, 1993; De Lisle *et al.*, 1993; Pavlik *et al.*, 1999; Quinn *et al.*, 2002).

## CONCLUSION

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.

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