

Evaluation of contamination of cattle's raw milk to Johne's disease in farms of Kaleybar regionYounes Anzabi^{1*}, Masoud Hassanzadeh Makoui², Behzad Abbasvand², Vahid Badiheh Aghdam³

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Abstract: Paratuberculosis or Johne's disease is a chronic disease that is untreatable in all ruminants and economically it is important. The clinical signs of Johne's disease lead to a decrease in the production and productivity of breeding animals. On the other hand, the important point is the possible role of bacteria causing the John disease in the pathogenesis of Crohn's disease in humans. The aim of this study was firstly to evaluate the characteristics of PCR technique on samples of raw milk and feces of cows to identify the positive cases infections of John's diseases (*Mycobacterium avium paratuberculosis*) in comparison with direct smear microscopy and second, to determine the bacterial contamination of milk produced in farms of Kaleybar region in order to understand the epidemiology of John's disease in cattle milk and its possible role of it in causing Crohn's disease in human. In this study, referring to 9 dairy traditional nomads in the region; a total of 69 dairy milking cattle were selected as the study population which had clinical signs of Johne's disease. The slides prepared from stool following special staining under Ziehl-Neelsen (ZN) and through bacterioscopy; clamping of acid-fast bacteria have been investigated in the developments and the number of positive and negative cases were identified. Then all stool and milk samples were immediately transferred to Biotechnology laboratory. These samples were centrifuged and then the DNA extraction was performed on sediment. In continue the extracted DNAs from each sample used as the template in the Thermal cycler machine for PCR. The results of this study showed that a total of 138 samples tested in PCR technique, 44 samples were positive containing 32% of total samples. Meanwhile of total 138 tested samples in direct microscopic examination only 28 samples showed positive response to the test. That is equivalent to about 20 percent of the total number of tested samples.

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1. Introduction

Paratuberculosis or Johne's disease is a chronic disease that is untreatable in all ruminants and economically it is important. The disease has a worldwide distribution and its prevalence is increasing in some countries. So far the outbreak of the disease in cattle in some provinces in the country have also been reported; although there are no accurate statistics of incidence and prevalence of the disease in cattle, sheep and goats in various parts of the country (Tabatabayi and Firouzi, 2001). Given the available evidence, disease in most parts of the country, especially in nomadic herds in Fars province is on the rise. The clinical signs of Johne's disease lead to a decrease in the production and productivity of breeding animals. On the other hand this untreatable disease will cause an irreparable damage to the country's farm system (Tabatabayi and Firouzi, 2001). The pathogen of this disease is *Mycobacterium avium paratuberculosis* which is present in contaminated milk, fecal and fetal bovine. However, the potential role of milk in the transmission of disease from mother to calf and

transfer of it to other traditional and industrial cattle farms has been recognized as one of the most important factors in development of this disease (Tabatabayi and Firouzi, 2001; Anzabi et al., 2005). On the other hand, the important point is the possible role of bacteria causing the John disease in the pathogenesis of Crohn's disease in humans. However, the main cause of Crohn's disease has not been expressed so far but many theories have been proposed about its bacterial origin and in this regard *Mycobacterium avium paratuberculosis* has a major role. Because, in various studies on the patients with Crohn's disease the bacteria have been isolated and identified and most of the antibiotics have been effective in Crohn's patients. These are the same compounds that have been used against *Mycobacterium avium paratuberculosis* as well (Anadolu et al., 1999).

The aim of this study was firstly to evaluate the characteristics of PCR technique on samples of raw milk and feces of cows to identify the positive cases infections of John's diseases (*Mycobacterium avium paratuberculosis*) in comparison with direct smear

microscopy and second, to determine the bacterial contamination of milk produced in farms of Kaleybar region in order to understand the epidemiology of John's disease in cattle milk and its possible role of it in causing Crohn's disease in human.

2. Material and Methods

In this study, referring to 9 dairy traditional nomads in the region; a total of 69 dairy milking cattle were selected as the study population which had clinical signs of John's disease (excessive weight loss, production loss, chronic diarrhea refractory to treatment). The slides prepared from stool and rectal wall (do it rectally) and following special staining under Ziehl-Neelsen (ZN) and through bacterioscopy; clamping of acid-fast bacteria (ZN-positive) have been investigated in the developments and the number of positive and negative cases were identified. In continue the samples were taken from cows and direct microscopic examination was performed on them. Then all stool and milk samples were immediately transferred to Biotechnology laboratory in School of Veterinary Medicine of Islamic Azad University of Tabriz Branch separately and in amounts required (at least five gram of feces and fifty milliliter of milk) and totally under sterile conditions and along with dry ice so that the PCR tests get performed in the samples. For this at first in biotechnology laboratory; these samples were centrifuged and then the DNA extraction was performed on sediment samples separately (Anzabi et al., 2005; Khakpoor et al., 2012). Following the extracted DNAs from each sample and according to a compiled program were placed in thermal device used as the template and the PCR components were added after. Finally, in electrophoresis level; the PCR products were electrophoresed in the tank followed by transfer on agarose gel. After gel electrophoresis test; results were evaluated and recorded. The details of each step are described as follows:

2.1. Extraction of DNA from available samples of our study

In this research, based on a method to extract DNA from samples sodium dodecyl sulphate (SDS) and proteinase (K) were performed. The two loops of stool and milk samples were centrifuged and the precipitate obtained in each case was mixed individually in sterile micro tubes with buffer TE (Tris, EDTA) and then were placed for 20 min at 80 °C in order to inactivate the bacteria present in the samples. Then the mixtures were placed for an hour at 37 °C nearby 50 micro liters Lysozyme and eventually we mixed it with a solution of SDS (10%). Then 10 microliter of a solution of 5molar NaCl were added to the micro tubes and while stirring; it was added in 80 microliter of a solution of CTAB/NaCl

(N-cetyl-N,N,N-trimethyl ammoniumbromide/NaCl) which is previously heated to 65 °C and then incubated for 30 min at 65 °C. After incubation of samples, a 75 microliter solution of chloroform / isoamylalcohol was added to micro tubes and was centrifuged for 8 minutes at around 11000g. Afterward 40 micro liter of Isopropanol were added to the micro tubes and the contents of micro tubes were stirred slowly until sometime after the nucleic acids were observed in the micro tubes. In this case, the micro tubes were transferred to - 20 °C freezer and were stored in this temperature for 3 minutes. Then the micro tubes were centrifuged in 12000g for fifteen minutes and the liquid of their top were poured out. Subsequently 1ml of cold Ethanol(70%) were separately added on the remaining deposits of each micro tube in freezer and the sediments were washed out in micro tubes by Vortex operation. Again the contents of micro tubes were centrifuged for 5 minutes at around 12000g and then the ethanol on top part of micro tubes were poured out and the resultant sediment which actually was pure extracted DNA were dried out in room temperature (Supply et al., 2001).

2.2. Performance of main stage PCR on DNA extracted from each sample

In this stage of the work, first by reference to the existing literature (Bhide et al., 2005; Pillai and Jayarao, 2002) and Gene bank along with investigation of IS900 chromosome sequence of Mycobacterium avium paratuberculosis (agent of John's disease); finally the primers were designed as follows and were prepared and used through Tuba Negin Company (Tehran, IRAN) (Anzabi et al., 2005; Khakpoor et al., 2012).

Forward Primer (FP-25) : CCA GGT TCG ACG GGG ATG GC
Reverse Primer (RP-25): GGT CGG TAC CCT CGG CGT CC

In order to perform the PCR test first all the extracted DNAs were separately mixed in sterile micro tubes and according to the mentioned protocol in following table with test's components (Khakpoor et al., 2012):

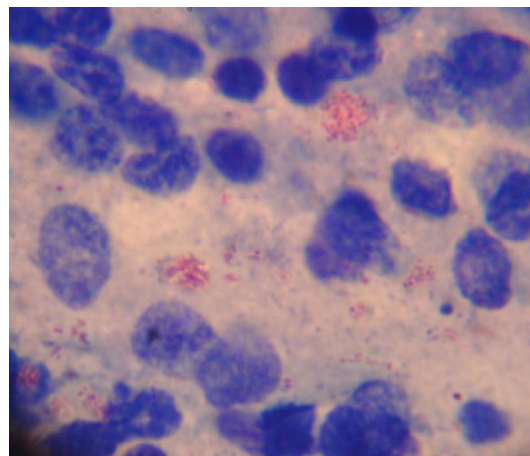
PCR content	amount	Final concentration
Sterile, redistilled H ₂ O	14.15µl	-
10x PCR buffer	2µl	Kcl: 50mM, Tris-C 120 µM
Mgcl ₂	0.6µl	1.5mM
dNTP mix	0.2µl	100µM
Forward primer	0.4µl	500 n M
Reverse primer	0.4µl	500 n M
TaqDNA polymerase enzyme (57µl)	0.25µl	1.25 U
Templet DNA	2µl	100ng
Total volume	20ml	

The micro tubes were placed inside thermal cycler machine of Ependorf Company and the selection of timing and temperature were as follows: first cycle, temperature 94 °C for 4 min for diffusion of DNA, in main cycles at 94 °C for 30 seconds for diffusion, at 70 °C for 30 seconds for attachment, at 72 °C for 40 seconds for operation and development and eventually in last cycle temperature of 72 °C for 7 min for final extension. It should be noted that above procedure were repeated for 35 times in the main cycles except in first and last cycles (Anzabi et al., 2005; Khakpoor et al., 2012) until the last step of PCR technique to determine the size of the PCR-products obtained from experiment in which the electrophoresis test was used. For this purpose first, the resulting product of PCR of each sample were transferred separately to the pre-prepared wells on 1% agarose gel and then using the 70 miliVolt AC power supply system and inside the special tank for gel, the operation of electrophoresis of the gels was performed (Khakpoor et al., 2012).

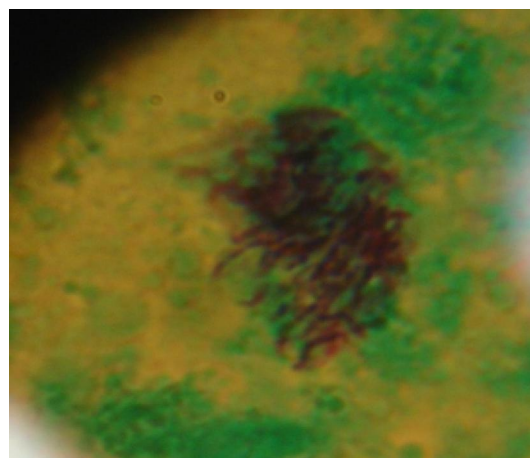
3. Results

In this study, from 69 sample of feces used for direct microscopic examination; 19 samples were reported positive which was equivalent to 27.5 percent of total samples and 50 samples were negative equaling to 72.5 percent of the whole sample. Moreover from the total of 69 tested milk samples 9 of them were positive which equals to 13% of the total samples among which 60 samples were negative corresponding to 87 percent of the total sample. On the other hand in direct microscopic examination; it was found that from 19 positive feces samples only 11 were PCR positive after the PCR testing comprising about 58% of positive feces samples in direct microscopic test. However, of the 50 negative feces tested samples in direct microscopic testing a total of 22 samples were also positive by PCR testing; this number is equivalent to 44% of feces samples which were negative in direct examination. Including all of the samples which were positive for fecal PCR test; it was found that about 48% of all feces samples of the test were successful. Also of 9 positive milk samples in direct microscopy which were selected for PCR testing one was negative but the other 8 samples were all positive, all of which contain about 89% of the number of positive samples of milk which examined by direct microscopy. Also, of 60 samples of milk tested negative on direct microscopy 3 other samples were PCR positive. This number equivalent to only 5 % of milk samples which were negative on direct

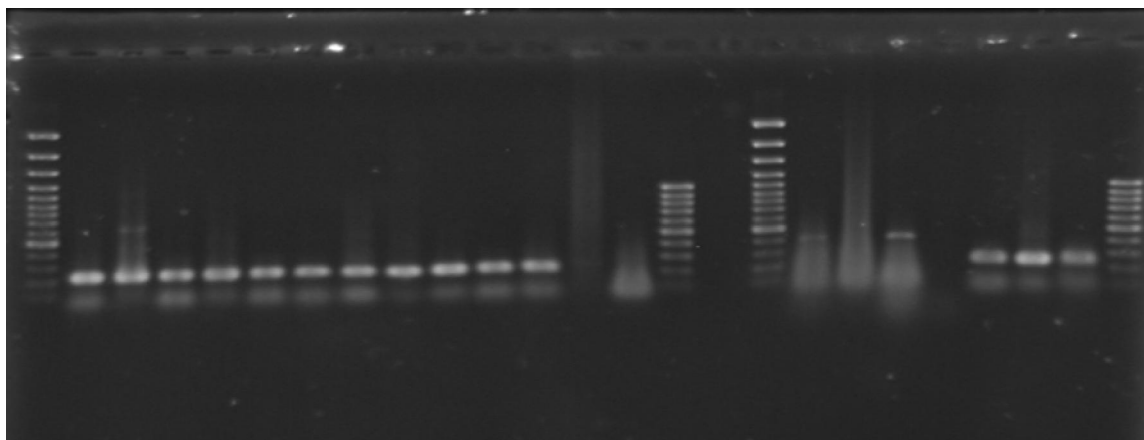
microscopic examination including all PCR positive samples of milk again, it was found in this study that about 16% of total 69 milk samples were PCR positive. Finally, the results of this study showed that a total of 138 samples tested in PCR technique, 44 samples were positive containing 32% of total samples. Meanwhile of total 138 tested samples in direct microscopic examination only 28 samples showed positive response to the test. That is equivalent to about 20 percent of the total number of tested samples.



Graph 1: Acid-fast Bailli, agent of Johne's disease in direct microscopic examination of samples of raw milk of cattle following ZN staining (Magnification: 400x).



Graph 2: Acid-fast bacilli cause of Johne's disease in clumping form; direct microscopic examination of feces samples from cattle following modified staining Zeihl-Neelsen (Magnification: 1000x).



Graph 3: a photograph of electrophoresis gel in PCR with primers' pairs FP-25 and RP-25 in rows 1 and 17 (from left to right) size marker Plus 100 bp from Fermentas company in Germany (respectively from top to bottom including bands as 3000, 2000, 1500,1200,1031,800,900,700,600,500,400,300,200,100bp) and in rows 15 and 25 size marker 100bp from Fermentas on Germany (respectively from top to bottom, including bands as 1031, 900, 800, 700, 500, 400, 300, 200, 100 and 80 bp) and in rows 3 to 12 and 22 to 24 product 228bp (positive) and the rows 19, 18, 14, 13, 20 the respond was negative and in rows 16 and 21 the control was negative and in row 2 the control was positive:

4. Discussion

The results of this study determines that the traditional cattle farms in Kaleybar region have the history of involvement with Johne's disease, physical symptoms as a screening marker for diagnosis of Johne's disease have had a better applications and PCR samples of milk in these animals as a confirmatory test does not match well with the clinical symptoms. This problem show inconsistency with the findings of Khakpour et al. that in Moghan region the pollution in cows' milk causing Johne's disease is in a relatively high agreement either among the symptoms of the disease or with findings of PCR tests (Khakpour et al., 2012). However, comparison of results in direct microscopic examinations and PCR testing of fecal samples from same animals are more consistent and significant in this perspective. It seems that one of the main reasons for the observed differences in the results of feces and milk samples often related to lower levels of bacteria removal through milk rather than through feces (Irene et al., 2002). On the other hand, presence of positive cases in the PCR test in term of negative samples of direct microscopy indicates that direct microscopy alone cannot serve as diagnostic test or gives a high confidence level in terms of epidemiology in study and diagnosis of Johne's disease because some of the negative cases in direct microscopic test in this study become positive in PCR test including 22.7% of mentioned samples. This finding is more consistent with results of Khakpour et al. who have expedited this amount to about 30% due to the nature of the intracellular pathogen of Johne's disease and development of chronic disease and even absence of

obvious symptoms in most cases. These results are predictable in other diagnostic procedures as well (Khakpour et al., 2012). To justify this matter; it also has been shown that in subclinical Johne's disease infection; a large number of bacteria causing the disease are in blood monocytes and tissue macrophages and it is possible that after some time they flush out. In this circumstance comparing to other methods of laboratory diagnosis; the PCR test will be able to better detect the presence of bacteria in the tested samples (Stevenson and Sharp, 1997). Study of literature shows that although most of the times for rapid diagnosis of Johne's disease, direct microscopic examination is used frequently but there are doubts about the sensitivity and specificity of this method and in most cases using it, the differentiation of agent of Johne's disease from other objects in acid-fast present in our tested sample is difficult (Lilenbaum et al., 2007; Zimmer et al., 1996). In this regard, even a number of reports have shown that the direct examination of fecal extensions after staining under Zeihl- Neelson is not a reliable diagnostic tool (Anzabi et al., 2009).

Numerous studies around the world testing has been performed using PCR test, often indicates that this technique is capable to record more positive samples in comparison with other methods of laboratory diagnosis. Also most of the researches show high sensitivity and specificity on this technique for diagnosis of Johne's disease. For example, in a study carried out in Italy and on Johne's disease in sheep, in the first phase; the sheep examined serologically and then serologically positive and negative samples were tested by PCR

technique of milk. 9 samples were related to 15 seropositive animals and 4 were for 14 serum negative cattle which were reported positive regarding milk PCR (Nebbia et al., 2003). Another study performed on water buffalo in India has shown that from 20 cases containing clinical symptoms 14 samples were positive with PCR testing and only 6 were positive in culture (Sivakumar et al., 2004). In another study which also has been done in India, in a comparative form; the sensitivity of different diagnostic methods for studying the Johne's disease have been studied. Among the test methods between cutaneous Ionian, tissue culture, feces culture, tissue PCR and ELISA, tissue PCR technique after culturing the tissue have demonstrated the best sensitivity (Tripathi et al., 2005).

Another study in year 2007 in India performed a comparison on the results of the three tests: ELISA, culture, milk PCR of local cows showed that milk PCR has offered the best results (Sharma et al., 2007). It should be noted that survey of studies on Johne's disease around the world shows that researchers have used tissue samples or even blood samples along with milk samples for PCR of samples. In a study carried out in Slovakia; buffy catgut was used as PCR sample to detect the Johne's disease (Bhide et al., 2005). In another research done by the author and colleagues in Tabriz; PCR tests on milk of 80 cows suspected of having Johne's disease, a total of 25 samples were positive (32% of tested cases). Moreover frequently positive cases have been reported among the apparently healthy and with pasteurized milk cattle which indicates a relatively high contamination of the region (Anzabi et al., 2005). But results of this study show 16% of contamination of raw milk of cows in Kaleybar region. This means contamination causing Johne's disease have been registered much lower in this district. It is worth mentioning the infection's rate in different regions of Iran and other countries have often been reported differently. Recent studies indicate that in dairy cattle in the U.S.A. nearly 40% of cattle are infected by Johne's disease (Pillai and Jayarao, 2002). Besides the studies performed in U.K. in case of contamination of pasteurized milk were 30% positive in PCR test (Millar et al., 1996). A study in year 1997 has showed that 60% of milk samples from 72 dairy cattle were positive in PRC test while the same samples were only 30% positive through bacteria culture (Stevenson and Sharp, 1997). Another important point that was found in the study was diagnosis of Johne's disease through examination of cows' feces showed much more positive results than examination of cows' milk samples both in direct microscopic test and in PCR test. These findings are consistent with results of

similar researches and in explaining of these differences they have announced that as the bacterium causing Johne's disease was so fastidious in growth stages of the bacterium and is severely associated with its growth factors therefore stability and excretion of the bacterium in milk was much lower than feces (Khare et al., 2004). It has also been announced in this regard that often the number of bacteria causing Johne's disease in milk of contaminated cows is considerably lower compared to feces (Khare et al., 2004; Pillai and Jayarao, 2002).

5. Conclusion

Finally considering the results of this study, indicating a relatively high rate of contamination in raw milk productions of Kaleybar region and the possible association between bacteria causing Johne's disease and Crohn disease in humans more attention is demanded in this case (Anadolu et al., 1999). On the other hand, the relatively high resistance of the bacteria to thermal treatments and its intracellular specificity; all contribute to the survival of these bacteria especially in pasteurization process of milk (Anzabi et al., 2005; Tabatabayi and Firouzi, 2001); requires more attention of researchers and food practitioners specially in health and quality control of nutrients.

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