ORIGINAL ARTICLE

Detection of *Mycobacterium bovis* in cattle suspected to tuberculosis by PCR method in Urmia-IRAN

Mohamad Hosein Sadeghi Zali[*1], Safar Farajnia [2], Hazhir Yahyapour[3], Tannaz Moslemzadeh[4] , Amin Hashempour[5]

1. Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad university of Urmia Branch, Urmia, Iran
2. Biotechnology Research center, Faculty of Medicine, university of Tabriz, Tabriz, Iran
3. Young Researchers and Elite Club, Sardasht branch, Islamic Azad University ,Sardasht ,Iran 4. Faculty of veterinary medicine, Islamic Azad university of Urmia Branch, Urmia Iran
5. Young Researchers and Elite Club, Urmia branch, Islamic Azad University ,Urmia ,Iran
Email: sadeghi.mohamadhosein@yahoo.com

ABSTRACT

Bovine tuberculosis (TB) is one of the most important zoonotic diseases worldwide. Cases of human tuberculosis of bovine origin have increased in recent Years, and this zoonosis has been become a public health problem. Mycobacterium bovis, the causative agent of bovine tuberculosis, infects both animals of agricultural importance and wild mammals, which act as a reservoir for the organisms, making it difficult to control the disease. The aim of this study was to detection of Mycobacterium bovis in blood and lymph nodes samples of cattle suspected to tuberculosis by PCR method. PCR assay amplifies a 500-bp fragment from the M .bovis genome by using the JB21-JB22 primer pair. Samples from cattle suspected to tuberculosis were initially decontaminated with the petroff method and ziehl – Neelsen staining was performed. Then specimens were checked by microscopic examination for acid fast bacilli and cultured on Lowenstein – Jensen medium containing sodium pyruvate. Specimens were also processed for PCR analysis. DNA was extracted from samples with CTAB and proteinase K method and entered in a PCR reaction using JB21-JB22 primers. The PCR products were electrophoresed on agarose gels and Visualized by Etidium Bromide staining. Of 100 suspected animals for Acid fast bacillus smear positive were 1(1%) sample , 2(2%) samples were culture positive and PCR was positive in 8(8%) samples . our results indicated that the JB21-JB22 PCR is a highly sensitive and specific method for Rapid diagnosis of infection caused by M.bovis and could be used for the diagnosis of infected animals .
Key words: Mycobacterium bovis, cattle, PCR

INTRODUCTION

Mycobacteria are rod-shaped bodies; acid-stable, non-motile, aerobic, non-spore and oxidative stresses that cause infections in humans and animals are important [1]. One of the major diseases caused by Mycobacterium tuberculosis and *Mycobacterium bovis* in cattle is caused besides that affects cattle. Humans and other animal species such as dogs, cats, sheep, goat, horse, badger and other mammalian species but also affects birds are resistant against these crimes [1, 21and23].Prevalence of bovine tuberculosis in terms of economic importance is important animal tuberculosis and because of joint disease and economic losses resulting from the chronic and progressive nature of the disease worldwide eradication program is running. Extent the situation in terms of livestock, cattle breed and health regulations in different countries [1, 2, 3, 14, 21and22] in recent years the number of patients with human tuberculosis of bovine origin has been rising [19]. Although bovine tuberculin test is an easy and effective way to identify infected animals, but not high sensitivity and specificity of this test is more sensitive and accurate are necessary(1, 14).For the diagnosis of bovine tuberculosis in live animals, we sputum, pleural fluid, cerebrospinal fluid, synovial fluid, blood, lymph node biopsy, fluid from the trachea and bronchi, or other questionable material to the preparation of the samples [1 and2].In livestock waste can also be prepared from samples offreshorfixedin10%formalincan be used, such as lymph

nodes(1).Prepared samples were first decontaminated and concentrated to be neutralized by the buffers are appropriate. The deposits obtained from various tests such as direct microscopic examination using the Zeil–Nielsen colors ,such as the selective agriculture Estein-Jensen containing sodiumpyruvate (4/0%) can be used [1, 2, 4, 15, 11, 18].The PCR method was used to study the papers, a sensitive, accurate and rapid detection of *Mycobacterium bovis* in the pathological specimens. JB21-JB22 primer pairs were designed in this study at 500bp with in a band to gel electrophoresis. The standard strain of *Mycobacterium bovis* (ATCC/19210) was used as a positive control. Genomic DNA samples were extracted using specific primers in the PCR reaction, the PCR product was agarose gel electrophoresis were ultimately positive controls were studied. According to the results of the experiments showed that the PCR method compared with other methods, the preferred method for the diagnosis of bovine tuberculosisis a disease.

MATERIALS AND MTHODS
In the present study a total of 100 head of cattle over the age of 1.5 Year Bovine TB was suspected that the sampling procedure was carried out. This research was conducted in the city of Urmia and the bulls and cattle suspected of suffering from impotence, decreased milk production efficiency and lower production and deployment ahead of Urmia slaughter house were collected from each of two samples of blood and other samples retropharyngeal lymph node samples.
The samples were transported to the laboratory and the DNA extracted from decontaminated and concentrated, and finally they were taken. Genomic DNA was extracted from each sample with specific primers (JB21, JB22) were inserted in the PCR reaction. Finally, the PCR products in agarose gel electrophoresis after PCR, and *Mycobacterium bovis* by PCR in the range of bands observed 500bp determined. In this study, the strain was used as positive control ATCC/19210 and local positive strains (20).
Lymph node preparations obtained from animals suspected of having bovine tuberculosis
The homogenization of samples of lymph nodes that need to be digested and disinfection in order to split the sample, save4% was added and samples were carefully crushed. The samples were mixed with gains for 1minandcentrifugedfor 30minin 3000rpmmoved.The resulting precipitate was added to the solution and the mixture was phenol Fetale in as PH indicator. HCL was added and mixed samples were then normalized.
The sample was homogenized with 7 = $_p$H and laboratory procedures such as painted and ready to be planted. For specific cell culture medium, the sample was homogenized in200Landais required.
Preparation of blood taken from animals suspected of having bovine tuberculosis
2ml of blood collected in tubes Venoject worked heparinised was poured in to a sterile test tube after mixing. Three times the volume of blood was transferred to tubes lysis buffer(1X) Lysis Buffer was added for 10minat 4° C, and the tubes were centrifuged for 15min, and the supernatant was discarded 2000rpm Steps to reach the platform (Pellet) white form was repeated. The pellets were placed in 80 ° C for 25min Durban Marie were also Fetaleinphenol solution and HCL was added.
DNA extracted from blood and lymph node
*The following substances were added to the sample preparation:
- SDS 10% -protein - CTAB / NaCL (1%) -phenol/chloroform-isopropanol-centrifuge-precipitation with ethanol 70%-TE buffer
In the present study, using data from the gene bank of M.bovis -specific primers for the latest in samples taken from cows with suspected bovine tuberculosis was used. UsingprimersJB21, JB22thatitsgenome sequence is as follows:
Primer JB21: 5'-TCGTCCGCTGATGCAAGTGC-3'
Primer JB22: 5'-CGTCCGCTGACCTCAAGAAG-3'
The product of these primers in PCR test piece is measured 500pb [20].

RESULTS
From 100 cows with suspected bovine TB samples from decontaminated and concentrated, microscopic tested using the Zeil–Nielsen colors and culture Estein-Jensen lacking glycerol and containing 4% pyruvate sodium were. All samples were then separately extracted genomic DNA was performed and the following results were obtained:
The direct microscopic examination using the Zeil–Nielsen colors 1 (1%), culture Estein-Jensen lacking glycerol and 4% pyruvate, 2 (2%) and PCR testing of buffy 2(2%) and Retropharyngeal lymph node8 (8%) were recorded in the positive for *Mycobacterium bovis*.
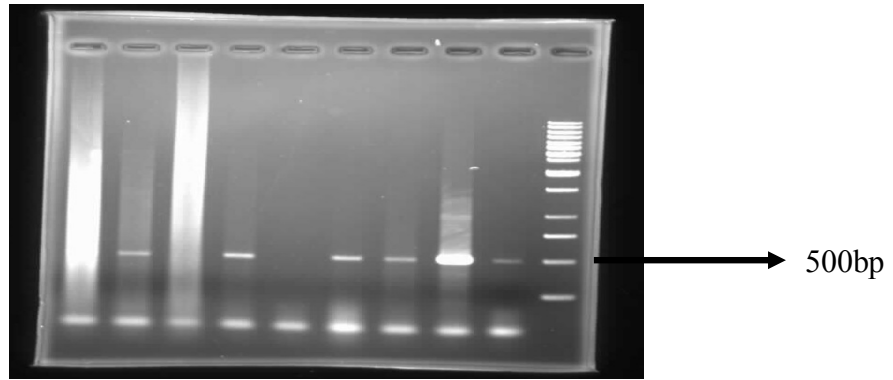
Picture1-electrophoresisof PCR tests on samples of retropharyngeal lymph node disease in cows with suspected bovine tuberculosis

-Columns10= 1kb DNA marker
-Columns2, 4, 6, and 7=positive examples of
-Column8= positive control of local strain
-Column9=sample standard positive control strain(ATCC/19210)
-Column5=negative control samples)

Picture2-Electrophoresis of PCR tests on blood samples of bovine tuberculosis in cattle infected

-Column9=Marker 1kbDNA
-Columns1 and5=positive examples of study
-Column7=positive control samples
-Column8=negative control samples
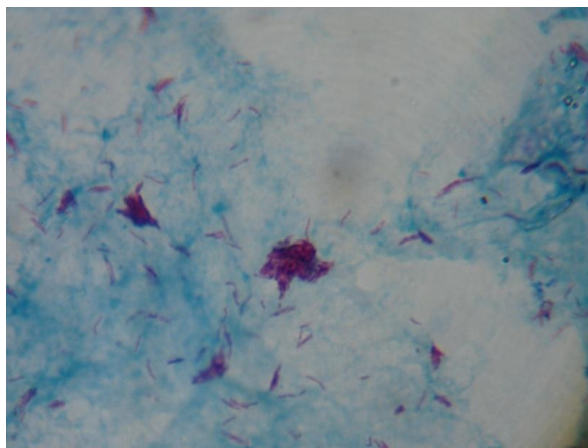
Picture3-Expand the preparation of samples positive for bovine tuberculosis in cattle infected with M. bovis using the following colors–Nielsen

Picture 4-Colonies grown in culture for *Mycobacterium bovis* Levine-Stein-Jensen containing sodiumpyruvate (4/0%).

DISCUSSION AND CONCLUSION

Mycobacteria cause infections in animals and humans and cause significant economic losses are. Bovine tuberculosisis caused by *Mycobacterium bovis* in human and animal health point of view is very important. The ease and frequency emissions cause tuberculosis from animals to humans, particularly in environments that are not under the control of bovine tuberculosis, a disease common to the disease could become an important track.

Agent of bovine tuberculosis infections in humans are caused by drinking contaminated milk. However, infection can also occur from breathing. Pasteurization of milk and the risk of infection can be almost completely eliminated. *Mycobacterium bovis* heat, drought and is resistant to most disinfectants, the mass of the heat, the humidity will remain alive for weeks [1, 8, and 10].

Although most cases of human tuberculosis due to *Mycobacterium bovis* Mycobacterium tuberculosis but also in human TB and in the United States 60 years ago, dairy herds infected with bovine bacilli were strongly and milk the animals, a common source of infection for bovine tuberculosis provided. Tuberculin test in cattle and killing those who had a positive reaction to the severity, prevalence of infection in cattle was reduced to less than 1 percent. Tuberculosis infection in countries where milk consumption is almost eliminated, however, until the complete eradication of the disease, *Mycobacterium bovis* should be considered in the differential diagnosis of tuberculosis [3].

Mycobacterium bovis causes TB spontaneously in a wide range of animals including cats, dogs and orders of mammals. Bacilliin the way humans usually through the digestive system the bovine tuberculosis lesions, mesenteric lymph nodes, especially in the pulmonary, neck, bones and joints are seen. *Mycobacterium bovis* is when the inhaled TB also makes it distinct from Mycobacterium tuberculosisis associated with pulmonary tuberculosis [3].

In this study from 100 male and female cattle older than5/1Year Bovine TB is suspected, and the thresholds were sent to slaughter, blood and action Retropharyngeal lymph node biopsy was performed under sterile conditions.

The samples were first decontaminated and concentrated using direct microscopy Zeil–Nielsen colors (ZN) and cultured on medium Estein-Jensen lacking glycerol and sodiumpyruvate (4%) groups. Morphology of *Mycobacterium bovis* bacilliin ZN approach taken in the development of red are seen in blue that Often compared to Mycobacterium tuberculosis bacilli are shorter and fatter than observed.

Mycobacterium bovis should also be used for the development of environments that are more rapid growth and thus the mass of sodiumpyruvate (4%) as a matter of choice Incentives growth medium should be increased other side of this organism. Unlike as Mycobacterium tuberculosis able to grow in the presence of glycerol as a substance inhibiting the growth of *Mycobacterium bovis* latter is a glycerol-free medium for the growth of these organisms should therefore be used. In terms of plant characteristics and nutritional requirements, and the slow growth rate of *Mycobacterium bovis* takes 3-8 weeks.

Appropriate incubation temperature of 37^0C. In aerobic conditions, the creamy fillings inners and raised with a rough center, which is easily dislodge the environment. If inners full of Mycobacterium tuberculosis from the rough and leathery and hard to be removed from the environment [2,3, 9, 14].

In this study, all samples taken from cows with suspected bovine TB testing Polymerase Chain Reaction(RCR)were studied as well as the standard strain mycobacterium bovis(ATCC/19210)strains as well as the place and Comparative were given. Primer pairs used in this studyJB21, JB22, which caused the band to be in the rangebp500gel electrophoresis and Bacterial DNA extraction of CTAB (N-cetyl-N, N-terimethyl ammouium bromide), a cationic detergent has been used. A recent material in terms of high-ion-protein complexes that are formed by adding chloroform and isopropanol, the complexes formed were isolated and pure bacterial DNA from samples be extracted.

To determine the sensitivity of the PCR machine to search for the standard strain of Mycobacterium bovis105-100Serialdilutionswere prepared from the PCR results in samples containing a concentration of bacteria105, 104,103, 102 (CFU) was positively evaluated in samples containing100(1 =100bacteria) and 101(10 =101bacteria) were negative. We study the sensitivity of the PCR machine at least 100 bacteria detected in samples tested. The results of the survey are included microscopic examination of stained using acid-stable in 1 (1%) was positive. This result may be due to the low number of mycobacteria in cattle waste hardly using the Zeil–Nielsen colors (ZN)approved and may be negative due to other positive examples recorded in other experiments, due to the small number of bacilli acid-is stable [14].

Also taking place in the culture medium of choice *Mycobacterium bovis* (medium Estein - Jensen containing sodium pyruvate (4%) without glycerol, only two cases (2%) of *Mycobacterium bovis* was identified that this could also be due to the incubation the result is a long, slow growing colonies of organisms that possible secondary infection or even a lack of essential nutrients is quite tangible. Although phenotypic detection methods and culture as a gold standard for the detection of *Mycobacterium bovis* yet known but according to new methods of diagnosis and the long incubation period for colony growth on the other, now this technique has been limited [1, 10].

The fastest ways to identify and search *Mycobacterium bovis* in samples taken from cows with suspected bovine TBPCR test is particularly molecular methods [4,17, 24].Barry et al; [7] using blood from cattle with bovine tuberculosis by PCR able to identify *Mycobacterium bovis* [7].

Shah et al (2002) using PCR and primers pncATB-1.2, pncAMT-2, JB21-JB22 able to differential diagnosis of Mycobacterium tuberculosis from *Mycobacterium bovis* [21] .Nassar and colleagues using primers JB21-JB22 in Brazil managed herds of dairy cattle with *Mycobacterium bovis* by PCR to isolate [12].

Rodriguez and colleagues in a study using PCR and primers JB21-JB22 with culture and conducted research on *Mycobacterium bovis* [19].

Araujo and colleagues in Caracas on 72 samples collected from cows with suspected bovine tuberculosis have been investigated. Their direct microscopic examination following staining - Nielsen 6/23 % of the samples and the method of PCR 5/76% of the samples were positive for the detection of *Mycobacterium bovis* [6].

Patel and colleagues PCR method to detect bovine tuberculosis could cow blood [13]. Retropharyngeal lymph nodes in the present study, blood samples taken from cows with suspected bovine TBPCR test were 8 cases (8%) Retropharyngeal lymph node and 2 (2%) samples were positive Buffy coat the polymerase chain reaction was recorded.

Because most of *Mycobacterium bovis* is localized in various organs, particularly the lymph nodes therefore, the presence of plaques in blood samples taken periodically, and may be a crime when there is no blood or PCR machine than its sensitivity to very small amounts of crime has not been able to identify the disease.

Bovine tuberculosis in humans due to its virulence role in public health and animal health in addition, it is also significant economic losses. According to the WHO tuberculosis infection *M. bovis* man in Brazil accounted for about 5% which gives better control of its transmission from cattle to humans implies. Outbreak of bovine tuberculosis in Brazil from 1989 to 1998 ,3/1% is estimated. Using PCR on biological

samples to reach a final diagnosis makes it possible, within 48 hours of the parasite to be identified. PCR testing methods sensitive, accurate and rapid identification of the parasite is *M. bovis* the samples [1, 16].

ACKNOWLEDGMENTS

REFERENCES
1. Hassani Tabatabaei. A, Firuzi. R., 2002. Bacterial Diseases of Livestock, Tehran University Press, Pages: 409-398
2. Rahimi. M. K., & Athari. A., 2004. Medical Microbiology Jawetz , translation , compilation: Guiv. F. Brooks, Janet S. Battle, Stephen. Morse, Ayizh, Pages: 468-451
3. Rahimi . M.. K. , 2004. Microbiology Zinsser, translation, compilation: Zhaclik et Ayizh , Pages: 200
4. Zahraei Salehi . T., &Shayegh . J.,2007. Veterinary Microbiology and microbial diseases (bacterial diseases). (Translation), Author: Quinn. BC. Jay. , Mark. B. K., Carter. M. E. , Tehran University Press, Pages: 146-133
5. Karimi. M., &Zeinali. S., 2004. Principles and Applications Laboratory PCR, (translation), Author: McPherson. M. J., Muller. S. J., Andisheye Zohor Press, Pages: 155 - 1
6. Araujo, c. p. , and et al. 2005. *Mycobacterium bovis* indentification by molecular method from post- mortem in spected cattle obtained in abattoirs of Mato Grosso do sul , Brazil , Mem inst oswaldo cruz , Riode Janeiro , vol. 100(7): 749- 752
7. Barry, T. Glennon, M., smith, T. & Gannon, F. 1993. Detection of *Mycobacterium bovis* in bovine blood by Combined PCR and DNA probe methods. Vet. Rec, 132:66-67
8. Banavalicer, J. N, Bhalotra, B., sharma, D. C, Goel, M. K., Khanndekar, P.S. & Bose, M. 1997. Identification of Mycobacterium Tuberculosis By PCR in clinical Specimens, Kingsway camp, Dellu- 110009
9. Carter, G. R., & Wise, D.J. 2004. Essentials of veterinary and Mycology, Iowa state press, pp: 207- 211
10. Hosek, J., Svastova,p., Moravkova,M., Pavlic, I. & Bartos, M. (2006):Methods of Mycobacterial DNA Isolation from different biological material, vet. Med, (5): 180- 192.
11. Monaghan, M.L., Doherty, M.L., Collins, J.D., kazda, J.F. & Quinn, P.J. 1994. The tuberculin test. Vet. Microbiology, (40): 111-124
12. Nassar, A.F.C., Miyashiro, S., oliveria, C.G., Pachpco, W.A. &Ogata, R.A. 2006. Isolation and identification of bovine tuberculosis in a Brazilian herd , Mem inst oswaldo cruz, Rio de Janeiro.
13. Patel, R.M.,Joshi,C.G., solanki, J.V. &Patel, P.R. 2001. PCR based detection of tuberculosis from Blood using oligo primers in cattle, Indian vet. 78: 480- 482.
14. Quinn, P.J., Markey, B.K.,Carter, M.E., Donnely, W. J. & Leonard, F.C.L. 2002. Veterinary Microbiology and Microbial Disease, Black well science Ltd, PP: 97- 105.
15. Quinn, P. J. , carter, M. E., Markey , B.M. &Carter, G.R. 1994. Clinical veterinary Micribiology. Wolfe published, PP:25- 156-157.
16. Ritelli, M., Amadori, M.,Tagliabue, S. & Pacciarini, M.L. (2003): use of a macrophage cell line for rapid detection of *Mycobacterium bovis* in diagnostic samples, vet. Microbiology, 94: 105- 120.
17. Romero, R. E., Garzon, D. L., Mejia, G.A., Monroy, W., Patarroyo, M.E & Murillo, L. 1999. Identification of *Mycobacterium bovis* in bovine Clinical samples by PCR Species- specific primers. Vet press, 63 (2): 101- 106.
18. Ronald, M. 2004. Microbiological Media, 3th edition, CRC press, PP: 1144- 1149.
19. Rodriguez, J. G., Fissanoti, J.C., Portillo, P.D., Pataroyo, M.E. , Romano , M.I. &Cataldi, A. 1999. Amplification of a 500- Base – pair Fragment from cultured isolates of Mycobacterium bovis, Journal of clinical Microbiology, P: 2330- 2332.
20. Rodriguez, J. G.,Mejia, G. A, Protillo, P.D., Patarroyo, M. E. & Murillo, L.A. 1995.Spicies- specific identification of *Mycobacterium bovis* by PCR, Microbiology, 141: 2131- 2138.
21. Shah. D. H., Verma, R., Bakshi,C.S. & singh, R.K. .2002. Amultiplex – PCR for the differentiation of *Mycobacterium bovis* and Mycobacterium tuberculosis, FEMS Microbiology Letters 214, PP:39- 43.
22. Songer, J. G., & Post, K.W. 2005.Veterinary Microbiology Bacterial and fungal Agents of Animal Disease, Elsevier Saunders, PP: 95- 101.
23. Timoney, J.F., Gillespie, J.H., Scott, F.W., & Barlough, J.E. 1988. Hagan and Bruner's Microbiology and Infectious Diseease of Domestic Animal, 8th edition. Comstock cornell, PP: 270- 289.
24. Yearsley, D. O' Rourke, J.,O' Brien, T. & Egan . J. 1998. Comparison of three methods for the isolation of Mycobacteria from bovine tissue Lessions. Vet Rec, 143, 480 – 481.