

DETECTION OF *MYCOBACTERIUM BOVIS* IN BUFFALOES BLOOD THROUGH POLYMERASE CHAIN REACTION (PCR) AND TUBERCULIN TEST

A. Khan, Z. I. Chaudhry, A. R. Shakoori, N. Mahmood, M. Ijaz, M. Z. U. Khan, Saima, I. Jan, and M. M. Ali

Department of Pathology, University of Veterinary and Animal Sciences, Lahore

ABSTRACT

A study was conducted in buffaloes to detect the *M. bovis* in blood samples through PCR. The PCR assay was described which could detect *M. bovis* infection in blood samples that were apparently negative in PPD of tubercle bacilli. A total of 100 buffaloes blood samples were tested and 2.0 % buffaloes were found positive for *M. bovis* infection through single comparative cervical intradermal tuberculin test (SCCIDTT) while 54 % were found positive for *M. bovis* infection through PCR. The positive cases were analyzed in the background of their history. History revealed that these positive buffaloes were reared in a populated herd for a long time. This study provides an evidence of *bovine tuberculosis* caused by *M. bovis* in buffaloes which are main source of milk and meat is South Asia.

Key words: bovine tuberculosis, buffaloes, prevalence, tuberculin test, PCR.

INTRODUCTION

Healthy and sound Livestock play a key role in the economy of a country. Riverine type buffalo of Pakistan belong to two breeds i.e. Nili-Ravi of Punjab and Kundi of Sindh province. Nili-Ravi buffaloes constitute approximately 79% of the total buffalo population in the country and are found in several parts of NWFP and AJK in addition to their primary home tract which is in irrigated Punjab. As per 1994 estimates, the population of buffaloes has increased to 19.7 million which is indicative of 5.04% annual growth rate since 1986. Pakistan has a population of over 32.4 million heads of cattle and 23.4 million of heads of buffaloes (Anonymous, 2006).

Buffalo is a flimsy animal and is easily exposed to various bacterial, viral and parasitic diseases that negatively affect the health of animal or may even prove fatal. Tuberculosis is an infectious, chronic, granulomatous, highly communicable, zoonotic, and debilitating disease. The aetiological agents of tuberculosis belong within the *M. tuberculosis* complex of bacteria (Rastogi et al, 2001), which comprises *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti* and *M. bovis* BCG (Behr, 2001), as well as *M. canetti* (Van Soolingen et al, 1997) and *M. caprae* (Aranaz et al, 1999). Interestingly, Kallenius et al. (1999) reported bacterial isolates with characteristics intermediate between *M. tuberculosis* and *M. bovis*. The bacteria in this complex are usually regarded as subspecies, having characteristically 99-9% similarity at the nucleotide level, with identical 16S rRNA sequences (Brosch et al, 2002). However, there are distinct phenotypic differences between these 'subspecies', not least their host range and pathogenicity. *Mycobacterium bovis* has the largest host range within the *M. tuberculosis* complex, and is capable

of infecting an extensive range of mammalian species including man (O'Reilly and Daborn, 1995).

Tuberculosis occur worldwide, in an extensive range of wildlife and farmed animals (O'Reilly and Daborn, 1995). Cattle can become infected in numerous ways, with animal age and behavior, existing environment and climate, and prevailing farm practices having significant influence. Inhaling *M. bovis* is the most probable and important route, as lesion distribution and pathology in field cases show predominant involvement of the upper and lower respiratory tract and associated lymph nodes (Pritchard, 1988; Crews, 1991; Corner, 1994; Neill et al. 1994; Whipple et al. 1996). Ingestion of *M. bovis* directly, e.g. from swallowing infected milk or from contaminated pastures, water or fomites is considered secondary to respiratory spread (Menzies and Neill, 2000). Genital transmission can occur also if the reproductive organs are infected, but this remains extremely rare (Neill et al. 1994), as are congenital and vertical transmitted infections. Infection by inhaling *M. bovis* bacilli, or possibly a single bacillus, in an aerosol droplet is a concept that is generally accepted (Neill et al. 1991). Tuberculosis affects 1.7 billion people/year which is equal to one-third of the entire world population. Tuberculosis is endemic in Pakistan with about 1.5 million people infected, and Pakistan ranks 6th among the 22 high-burden tuberculosis countries worldwide.

According to the World Health Organization (WHO), Pakistan accounts for 43 % of the TB disease burden in the WHO Eastern Mediterranean Region. Every year, approximately 270,000 people in Pakistan develop TB, with nearly 80 % occurring among young people aged between 15 and 49, although the TB cure rate in Pakistan is some 78 %. Bovine TB is still a significant zoonosis in many parts of the world and it accounts for 25.8% of TB in man. *M. bovis* is the major

cause of human gastrointestinal TB in the developing countries where bovine milk had not often been pasteurized before use (Bonsu et al. 2000). Milk-borne infection is the main cause of non-pulmonary tuberculosis in areas where bovine tuberculosis is common and uncontrolled (Daborn et al. 1996). The literature surveyed showed no record of such work over bovine tuberculosis due to *M. bovis* in buffaloes in Pakistan. Hence, the present study has been designed with following objectives; Comparison of various diagnostic tests (tuberculin test and PCR) in animals; standardize the tuberculin test in buffaloes and to identify infected animals; detect *M. bovis* in blood of buffalo and to standardize a molecular based PCR method for diagnosis of *Mycobacterium bovis*.

The reason behind is the absence of a single rapid test that clearly diagnoses and detect the *M. bovis* infection in buffaloes. Keeping in view these all facts and importance of the disease to be eradicated the present study has been designed.

MATERIALS AND METHODS

Selection of Experimental Animals: For screening and sampling of bovine tuberculosis, a total of 100 buffaloes of different ages were selected from different Cattle Colonies at Band road (i.e; Shafeeq abad, Gao shala, Bijli ghar and Kokar Pind) Lahore. The blood samples collected from 100 buffaloes were labeled according to their local names. The blood samples were brought to the Department of Pathology, University of Veterinary and Animal Sciences, Lahore in ice box and were kept at -70°C.

Tuberculin Test: Single Comparative Cervical Intradermal Tuberculin Test (SCCIDTT) was used for screening animals. Both mammalian and avian PPD-Tuberculin (Purified Protein Derivative of tubercle bacilli) was obtained from Veterinary Research Institute, Ghazi Road, Lahore, in ampules of 50 doses each. Preparation of the animal for test, test procedure and recording of the results were done according to the method used by Hamid (2002) and as per instruction issued by the Biological Production Division, Veterinary Research Institute, Ghazi road, Lahore, on the use of PPD tuberculin.

DNA Isolation: DNA was isolated with the help of GENTRA PUREGENE®, USA. DNA Purification Kit. The isolated DNA was analyzed by 0.7 % agarose gel electrophoresis to confirm the presence of isolated genomic DNA.

Polymerase Chain Reaction: PCR method adopted was same as described by (Prabhakar et al. 2004) with some modifications. The primer sets used in PCR are described in (Table 1). 50 µl PCR reaction mixture contained 5 µl of 10X PCR buffer, 5µl of 2.5 mM dNTPs, 0.5µl of AmpliTaq DNA polymerase, 2µl of forward primer (100 pmol/µl), 2µl of reverse primer (100 pmol/µl), 5µl of 25 mM MgCl₂, 5µl of DNA sample and 25.5µl of distilled water were used for target gene amplification in PCR reaction mixture. PCR reaction mixture tubes were kept in thermal cycler (Applied Biosystems) and programmed with initial denaturation at 94 °C for 4 min. coupled with 30 cycles comprising 93 °C for 30 sec., 50 °C for 30 sec., 72°C for 30 sec. and final extension for 7 min. at 72°C. The amplified DNA fragments were analyzed after electrophoresis on 1 % Agarose gel.

Table I. PCR based detection of the *Mycobacterium bovis*.

Primers	Primer Sequences	Product size	Target region
JB21(F)	5'TCGTCCGCTGATGCAAGTGC3'	500 bp	hupB gene
JB22(R)	5'CGTCCGCTGACCCTCAAGAAG3'	500 bp	

RESULTS AND DISCUSSION

Mycobacterium bovis is major cause drug resistant tuberculosis in developing countries (Cosivi et al. 1998). The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries. However, with reports of tuberculosis due to *M. bovis* in AIDS patients (O'Reilly et al. 1995) and with increasing incidence of tuberculosis globally, proper differentiation of *Mycobacterial* strains, their route of transmission and rapid and reliable diagnostic assays are required for detection and identification of the pathogenic

mycobacteria in blood samples. This is essential for the rapid diagnosis, treatment, and control of tuberculosis.

The present study was designed to determine the efficacy of Polymerase Chain Reaction (PCR) in comparison with tuberculin test and to standardize the tuberculin test in buffaloes and to detect *Mycobacterium bovis* in the blood sample of same through PCR. A total of 100 buffaloes of different ages were selected from different Cattle Colonies at Band road (i.e; Shafeeqabad, Gaoshala, Bijli ghar and Kokar Pind) Lahore. The Single Comparative Cervical Intradermal Tuberculin Test (SCCIDTT) was standardized in these buffaloes. Under aseptic condition blood samples were also collected from

the same buffaloes. The blood samples were examined for the presence of *Mycobacterium bovis* by adopting PCR strategy.

It was found that out of 100 samples, 54 samples (54 %) were positive by PCR for *Mycobacterium bovis*.

Amplified DNA was detected by running PCR products on 1 % agarose gel that produced 500 bp bands by using primers JB21(forward) and JB22 (reverse) (Figure 1,2). Results are shown in percentage in figure 3.ibt

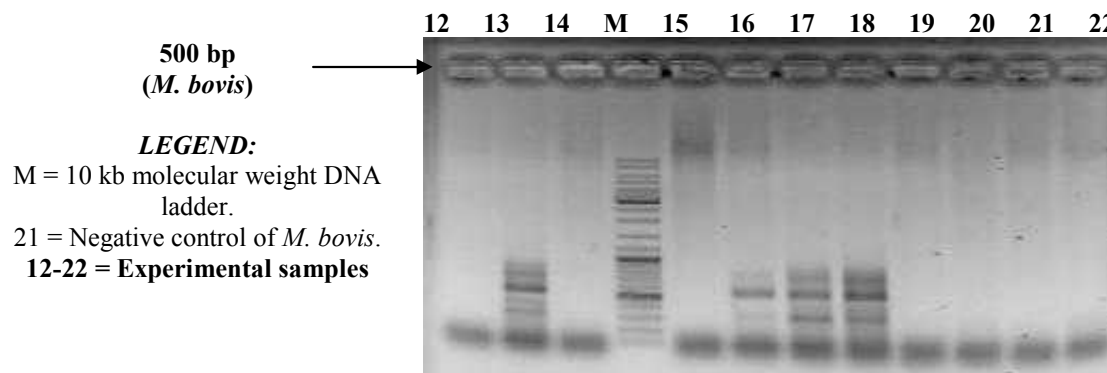


Figure 1. Representative photograph indicating the detection of *M. bovis* in buffalo's blood.

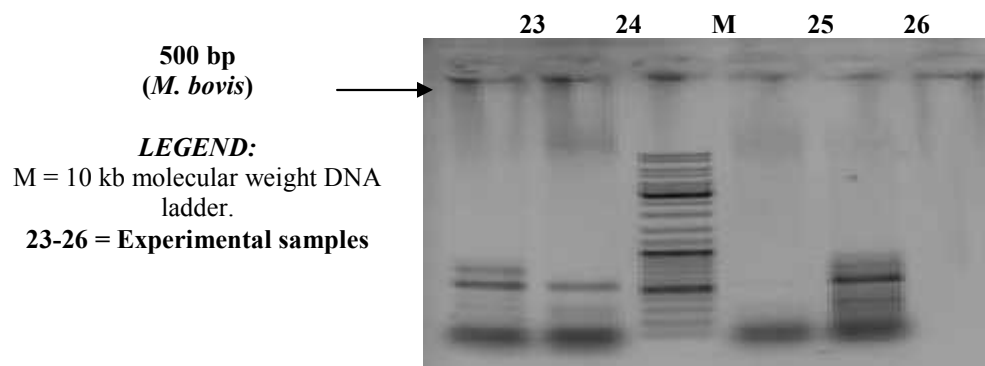


Figure 2. Representative photograph indicating the detection of *M. bovis* in buffalos blood.

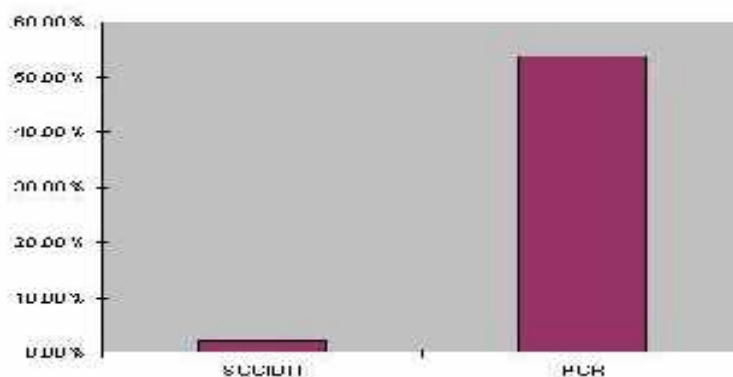


Figure 3. Histogram showing the comparative results of Tuberculin Test and Polymerase Chain Reaction (PCR) for the detection of *Mycobacterium bovis* in buffaloes blood.

Results indicated that 2.0% and 54% of buffaloes were found positive for *M. bovis* infection with the tuberculin test and polymerase chain reaction (PCR) respectively. From the results it is evident that polymerase chain reaction (PCR) was more sensitive than the tuberculin test for the diagnosis of bovine tuberculosis

in buffaloes and give much higher percentage of positive cases (54%) as compared to tuberculin test which revealed a positive percentage of 2.0%.

In this study 500 bp fragment amplified by using JB primer set specific for identification of *M. bovis*. A PCR assay is standardized to detect *M. bovis* on the basis

of amplicon size. The JB primer set used in PCR provided desired size amplification (500 bp) at T_m 50 °C by keeping other reagents conditions constant. The other two T_m conditions viz., 60 °C and 67 °C were also checked and no amplification was observed at these two conditions. However these results are not exactly matched with Shah et al. (2002) who reported the T_m value of 67 °C for the amplification of 500 bp fragments from *M. bovis*. The JB primer set targets hup-B gene locus as a result the desired size fragment was amplified. The recent research finding is also in agreement with Prabhakar et al. (2004), who also identified the hup-B gene and successfully used it to detect and differentiate *M. bovis* from other *Mycobacteria* in clinical samples. Single-step PCR procedures to differentiate *M. bovis* from *M. tuberculosis* by using IS6110 (Hellyer et al. 1996) alone or in association with *mtp40* gene have yielded discrepant results (Clarridge et al. 1993). The JB primer set is specific to *hupB* gene which is not located in the RD loci. The sequencing project has revealed that the genome of *M. bovis* is >99.9% identical to *M. tuberculosis*. However, *hupB* is present in every isolate of *M. tuberculosis* and *M. bovis*.

In this study 100 buffaloes were selected for tuberculin test and blood samples were also collected from same buffaloes. Out of these 100, two buffaloes were positive for *M. bovis* infection screen out through tuberculin test and fifty four out of 100 blood samples were positive for *M. bovis* in PCR test. The low percentage regarding tuberculin test was attributed to low quality formulation or impurities in tuberculin. DNA amplification by PCR provides a rapid and sensitive method for the detection of *M. tuberculosis* complex from clinical samples. The PCR proposed in this study was based on one-step amplification of one *mycobacterial* genomic DNA fragment amplication specific for *M. bovis* which is of 500-bp size. All the the DNA of *mycobacteria* extracted from the blood samples of buffaloes were subjected to amplification by PCR assay containing primer combination JB21, JB22. As expected, a unique 500-bp amplified product was obtained, using DNA from *M. bovis*. Interestingly, 14 (54%) out of 26 blood samples isolates tested revealed a 500-bp product similar to that observed with *M. bovis*. The PCR could successfully detect *M. bovis* from *M. tuberculosis* strains as in all the cases a single product corresponding to 500 bp was detected in *M. bovis*. The method was highly specific as none of the *mycobacterial* species other than *M. bovis* gave any amplification signal. When the agarose gel electrophoresis was used for detection, the method was able to detect 20 pg of pure DNA (4000 genome equivalents) from *M. bovis*. Therefore, this PCR assay could be a very useful tool for the rapid detection of *M. bovis*.

The similar observations were observed by Soilingen et al. (1994), who presented that in Argentina,

most human *M. bovis* infections are due to transmission from cattle. Fritsche et al. (2004) reported a case of tuberculosis in cattle exposed to a patient infected with *M. bovis*, where the strain isolated in the cattle and the patient were identical. As the patient is reported to have been exposed and contaminated during childhood, this seems to be the first documented case of transmission of *M. bovis* from animal to man and back to animal. In the present study it was observed that the current PCR assay is sensitive and have good ability to differentiate different strains of the *mycobacteria* as compared to other target genes such as IS6110, MTP 40, IS1081, IS990 etc., which have limitations in detection and differentiation of *mycobacterial* family members. 500 bp fragment amplified by JB primers is comparatively better target gene for detection and differentiation of *mycobacterial species* in clinical samples, especially for the detection and differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* in clinical samples of animals.

The present study regarding the detection of *M. bovis* in buffalo blood has significant importance because there is no any other report regarding the prevalence of *M. bovis* in blood of local buffaloes. The study has also importance because man is also dependent on buffaloes regarding meat and milk requirements so for better health, meat and food devoid of any pathogen is basic prerequisite. In future in order to enhance the quality of animals and animal food products we can exploit the finding of recent research.

Acknowledgments: This study was supported by Higher Education Commission of Pakistan. Authors are highly thankful to the dairy farmers' cooperation for offering their farms animals for this experiment.

REFERENCES

- Anonymous, (2006). Epidemiology of major livestock diseases in Pakistan. Annual Report for the year, 1992-93. Disease Investigation in Laboratory, Quetta.
- Aranaz, A., E. Liebana, E. Gomez-Mampaso, J. C. Galen, D. Cousins, A. Ortega, J. Bla'Zquez, and F. Barquero (1999). *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *M. tuberculosis* complex isolated from goats in Spain. *Int. J. Syst. Bacteriol.* 49: pp. 1263–1273.
- Ayele, W. Y., J. Neill, J. Zinsstag and M. G. Weiss (2004). Bovine tuberculosis: an old disease but a new threat to Africa. *Int. J. Tuberc Lung Dis.* 8: pp. 924-937.
- Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane and P. M. Small (1999). Comparative genomics of BCG vaccines by

- whole-genome DNA microarray. *Science*. 284: pp. 1520–1523.
- Bonsu, O. A., E. Laing and B. D. Akanmori (2000). Prevalence of tuberculosis in cattle in the Dangme-west district of Ghana, public health implications. *Acta Tropica*. 76(1): pp. 9-14.
- Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, M. Parsons, A. S. Pym, S. Samper, D. V. Soolingen and S. T. Cole (2002). A new evolutionary scenario for the *M. tuberculosis* complex. *Proc. Natl. Acad. Sci. USA*. 99: pp. 3684-3689.
- Clarridge, J. E., R. M. Shawar, T. M. Shinnick and B. B. Plikaytis (1993). Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J. Clin. Microbiol.* 31: pp. 2049-2056.
- Corner, L. A. (1994). Post mortem diagnosis of *Mycobacterium bovis* infection in cattle. *Vet Microbiol.* 40: pp.53–63.
- Cosivi O., J. M. Grange, C. J. Daborn, M. C. Raviglione, T. Fujikura, D. Cousins, R. A. Robinson, H. F. A. K. Huchzermeyer, I. D. Kantor and F. X. Meslin (1998). Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerg. Infect. Dis.* 4: pp. 59-70.
- Crews, K. B. (1991). Post-mortem findings in bovine tuberculosis reactors. *Surveillance*. 18: pp. 15–16.
- Daborn, C. J., J. M. Grange and R. R. Kazwala (1996). The bovine tuberculosis cycle – an African perspective. *J. Appl. Bacteriol. Suppl.* 81: pp. 27–32.
- Fritsche, A., R. Engel, D. Buhl and J. P. Zellweger (2004). *Mycobacterium bovis* tuberculosis: from animal to man and back. *Int. J. Tuberc. Lung. Dis.* 8: pp. 903.
- Hamid, J. P. and A. Suleman (2003). Bovine tuberculosis in dairy animals at Lahore_a threat to public health. Metropolitan Corporation, Lahore, Pakistan.
- Hellyer, T. J., L. E. Desjardin, M. K. Assaf, J. H. Bates, M. D. Cave and K. D. Eissenach. (1996). Specificity of IS6110-based amplification assay for *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* 34: pp.2843-2846.
- Kallenius, G., T. Koivula, and S. Ghebremichael (1999). Evolution and clonal traits of *M. tuberculosis* complex in Guinea-Bissau. *J. Clin. Microbiol.* 37: pp. 3872–3878.
- Menzies, F. D. and S. D. Neill (2000). Cattle-to-cattle transmission of bovine tuberculosis. *The Veterinary J.* 160: pp. 92–106.
- Neill, S. D., J. J. O'brien and J. Hanna (1991). A mathematical model for *Mycobacterium bovis* excretion from tuberculous cattle. *Vet Microbiol.* 28: pp. 103–109.
- Neill, S. D., J. M. Pollock, D. B. Bryson and J. Hanna (1994). Pathogenesis of *Mycobacterium bovis* infection in cattle. *Vet Microbiol.* 40: pp. 41–52.
- O'rielly, L. M. and C. J. Daborn (1995). The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. *Tuber Lung Dis.*, 76(1): pp. 1-46.
- Prabhakar, S., A. Mishra, A. Singhal, V. M. Katoch, S. S. Thakral, J. S. Tyagi and H. K. Prasad (2004). Use of the hupB Gene Encoding a Histone-Like Protein of *Mycobacterium tuberculosis* as a Target for Detection and Differentiation of *M. tuberculosis* and *M. bovis*. *J. Clin. Microbiol.* 42(6): pp. 2724–2732.
- Pritchard, D. G. (1988). A century of bovine tuberculosis 1888–1988 – conquest and controversy. *Comp Pathol.* 99: pp. 357–399.
- Rastogi, N., E. Legrand and C. Sola (2001). The *Mycobacteria*: an introduction to nomenclature and pathogenesis. *Rev. Sci. Tech. Off. Int. Epi.* 20: pp. 21–54.
- Shah, D. H., R. Verma, C. S. Bakshi and R. K. Singh (2002). A multiplex-PCR for the of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *FEMS Microbiology differentiation Letters.* 214: pp. 39-43.
- Soolingen, D., P. E. De-Haas, J. Haagsma, T. Eger, P. W. Hermans, V. Ritacco, A. Alito and J. D. V. Embden (1994). Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. *J. Clin. Microbiol.* 32: pp. 2425-2433.
- Soolingen, D. V., T. Hoogenboezem and P. E. De Haas (1997). A novel pathogenic taxon of the *M. tuberculosis* complex, Canetti: characterisation of an exceptional isolate from Africa. *Int J Syst Bacteriol.* 47: pp.1236–1245.
- Whipple, D. L., C. A. Bolin and J. M. Miller (1996). Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J. Vet. Diagn. Invest.* 8: pp.351–354.
- WHO. 2004. Tuberculosis: Infection and transmission, media centre, World Health Organization Fact sheet No. 104.