

Reliability of PCR for Detection of Bovine Tuberculosis in Pakistan

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Abstract.- PCR was used to detect *Mycobacterium tuberculosis* (MTB) complex and *Mycobacterium bovis* in local bovine milk samples. Along with PCR a single intradermal tuberculin test was also carried out for comparing the PCR with tuberculin test. In the case of MTB complex, a 984 bp fragment of insertion sequence *IS6110* was amplified in TB positive cases while for *M. bovis* a 500 bp genomic fragment was amplified in milk samples. The multiplex PCR was also done by using both sets of primers specific to MTB complex and *M. bovis*. The incidence of MTB complex and *M. bovis* was found to be 11(35%) and 9(29%) respectively by PCR in milk samples out of total 31 samples, collected from buffaloes suspected for TB, as against 3 (9.6 %) cases of bovine TB after single intradermal tuberculin test.

Key words: Bovine tuberculosis, tuberculin test.

INTRODUCTION

Bovine tuberculosis (TB) is widespread throughout world and, despite its name, infects a variety of hosts, including wildlife, domestic livestock and humans. Bovine TB is caused by *Mycobacterium bovis*, a Gram-positive bacillus with zoonotic potential that is genetically related to *Mycobacterium tuberculosis*, the causative agent of human tuberculosis (O'Reilly and Daborn, 1995; Cole *et al.*, 1998). One-third of the world's human population is infected with *M. tuberculosis*, and 3 million human deaths annually are attributable to the organism (Acha and Szyfres, 1987; Cornejo *et al.*, 1998).

The pulmonary form of zoonotic tuberculosis (TB) caused by *M. bovis* in humans is indistinguishable from the TB caused by *M. tuberculosis* (strict sense). However, *M. bovis* considered the ancestral variant of the other species of mycobacterium tuberculosis complex has the widest host range including animals and humans. Hence, animal TB poses a potential threat as a source of TB in humans. In countries where bovine TB is uncontrolled, most human cases occur in young persons and result from the drinking or handling of contaminated milk or milk products

0030-9923/2008/0005-0347 \$ 8.00/0

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(Acha and Szyfres, 1987; Cosivi *et al.*, 1998; Dankner *et al.*, 1993; Sreevatsan *et al.*, 1997).

From a review of a number of zoonotic tuberculosis studies, published between 1954 and 1970 and carried out in various countries around the world, it was estimated that the proportion of human cases due to *M. bovis* accounted for 3.1% of all forms of tuberculosis: 2.1% of pulmonary forms and 9.4% of extra pulmonary forms (Gervois *et al.*, 1972). In Indo-Pakistan subcontinent the incidence of bovine tuberculosis in cattle and buffalo was nearly 2.25% of the population in 1969 (Lall, 1969) and 10% in 1975 (AVA, 1975). It was reported to be 7.3% in cattle and buffaloes being slaughtered at Lahore abattoirs in 1989 (Shahid, 1989). According to a rough estimation, 20% patients of tuberculosis visiting various hospitals of Lahore are suffering from extra pulmonary tuberculosis. In some countries, human disease caused by *M. bovis* is merely reported as TB to avoid inquiries from disease control agencies, which might generate problems of patient confidentiality (O'Reilly and Daborn, 1995).

Currently, animal TB is diagnosed by a skin test that includes a caudal fold, comparative cervical, single cervical, or double-strength cervical test. The skin test has a low sensitivity (65.6 to 70%). Although some reports suggest that skin tests have a high specificity (98.8%), false-positive

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results due to exposure to atypical mycobacteria, corynebacteria, *Fasciola hepatica* (liver fluke), and/or nocardia species are problematic in some countries (Acha and Szyfres, 1987).

In this study PCR based detection was carried out for MTB complex and *M. bovis* in milk samples and compared with the conventional tuberculin test.

MATERIALS AND METHODS

For this study 31 milk samples were collected from buffaloes suspected for TB clinically, present in cattle colonies and different dairy farms found in periurban areas of Lahore, Pakistan. Milk samples (50 ml each) were collected in sterile pre-cooled McCartney bottles and stored at -20°C.

Single intradermal tuberculin test

All 31 samples were then subjected to Single Intradermal Tuberculin Test (SITT) as described in Guidelines from the World Organization for Animal Health (OIE, 2004). Mammalian purified protein derivative of tubercle bacilli (PPD) was obtained locally from Veterinary Research Institute, Ghazi Road Lahore, Pakistan.

Multiplex PCR for MTB complex and M. bovis

The DNA isolated from all 31 bovine milk samples by using GENTRA PUREGENE®, DNA Purification Kit USA were subjected to multiplex PCR by using two sets of primers: The first set comprised of forward IS5 (CGGAGACGGTGCGTAAG) and reverse IS6 (GATGGACCGCCAGGGCTTGC) primers amplifying a 984 bp genomic fragment of insertion sequence *IS6110* specific for MTB complex. The conditions were used as described by Barouni *et al.* (2004). The second set contained forward JB21(TCGTCCGCTGATGCAAGTGC) and reverse JB22(CGTCCGCTGACCTCAAGAAAG) primer amplifying a 500 bp genomic fragment specific for *M. bovis*. The duplex PCR reaction was performed in a final volume of 50 µl containing 1× PCR buffer (Fermentas), 2.5 U of *Taq* polymerase (Fermentas), 0.2 mM each deoxynucleoside triphosphate, 4.0 mM Magnesium chloride, and 150 pmol of first primer set and 100 pmol of second primer set. The target

DNA denatured by incubation for 4 min at 94°C before amplification for 30 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. The final extension was carried out at 72 °C for seven minutes. All reactions were carried out in an automated thermal cycler (Eppendorf, USA). After amplification, the PCR mixture was analyzed by gel electrophoresis in 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide.

PCR for Mycobacterium bovis

The extracted DNA from all 31 samples of bovine milk were also subjected to PCR by using single set of primers containing forward JB21 (TCGTCCGCTGATGCAAGTGC) and reverse JB22 (CGTCCGCTGACCTCAAGAAAG) amplifying a 500 bp genomic fragment specific for *M. bovis* as also described by Rodríguez *et al.* (1999) and Shah *et al.* (2002). The reaction was performed in a final volume of 50 µl containing 1× reaction buffer (Fermentas, USA), 2.5 U of *Taq* polymerase (Fermentas, USA), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM Magnesium chloride, 2 µg of each DNA and 100 pmol of each primer. Target DNA was denatured by initial incubation for 4 min at 94°C before amplification for 30 cycles of 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 sec and final extension at 72°C for 7 minutes. All reactions were carried out in an automated thermal cycler (Eppendorf, USA). After amplification, the PCR mixture was subjected to 1.5 % agarose gel electrophoresis.

RESULTS AND DISCUSSION

Tuberculin test

Table I shows the results of tuberculin and PCR tests of 31 blood samples. Out of 31 animals, only three were confirmed positive (Table I). This confirmation was based on the thickness of skin (4 mm or above) and localized skin reaction after 72 hours at the site where mammalian tuberculin was injected. There were only two doubtful cases as the skin thickness was between 2-4 mm after 72 hrs. Twenty six (26) animals showed no indurations or

swelling of skin below 2 mm were considered as negative for bovine tuberculosis.

Multiplex PCR

Out of 31 blood samples collected from buffaloes, 11 (35 %) were found positive for MTB complex, amplified at 984 bp (Fig. 1) and 09 (29%) were positive for *M. bovis*, amplified at 500 bp (Figs. 1, 2; Table I).

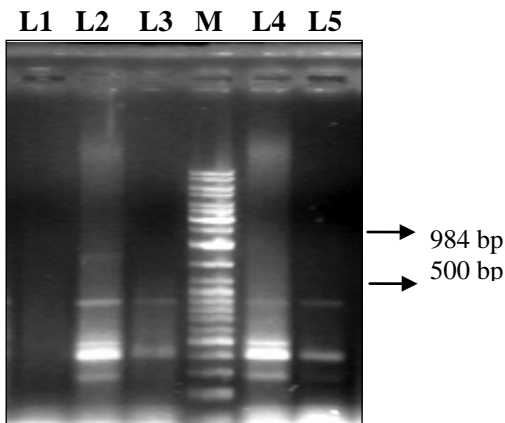


Fig 1. Multiplex PCR showing 984 bp product of MTB Complex and 500 bp product of *M. bovis*, in lanes L1-L5, obtained by using primers specific for MTB complex and *M. bovis*. M is DNA Marker.

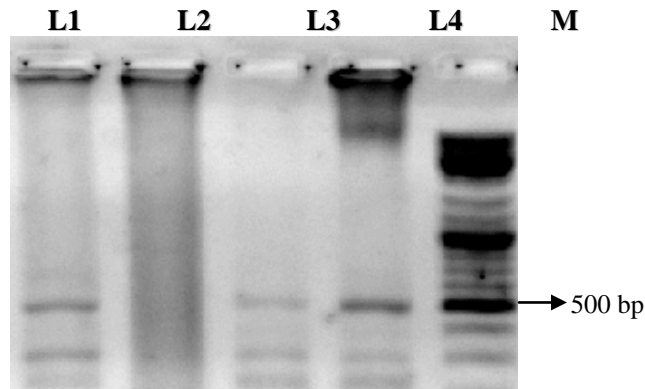


Fig. 2. A 500 bp PCR product (L1, L3, L4) of *M. bovis*, obtained by using JB21 and JB22 primers in a single PCR. L2: no amplification. M, DNA marker.

In Pakistan tuberculosis is prevalent at epidemic proportions, in animals, humans and maybe in the wildlife. The incidence of bovine

tuberculosis is 6.91% in buffaloes and 8.64% in cows (Suliaman *et al.*, 2002). The single intradermal tuberculin test (SITT) with bovine tuberculin purified protein derivative (PPD) is the OIE recommended test for screening against TB. The bovine PPD is a protein derivative extracted from *M. bovis*.

Table I.- Results of Tuberculin test applied on 31 buffaloes and PCR of 31 milk samples obtained from same buffaloes.

Sample No.	Tuberculin Test	PCR for MTB complex	PCR for <i>M. bovis</i>
1, 2, 4, 6-12, 16-18, 23, 24, 26-27, 29	—	—	—
3	+	+	+
5	+	+	+
13	±	+	+
14	—	+	+
15	+	+	+
19	±	—	—
21	—	+	+
22	—	+	+
25	—	+	—
28	—	+	+
30	—	+	—
31	—	+	+

Note: +, Positive; —, negative; ±, Doubtful

In this present study out of 31 buffaloes suspected for bovine tuberculosis showing the clinical signs, 3(9.6%) were positive for SITT, two were doubtful, whereas remaining were negative to SITT (Table I). The observations were made after every 24, 48 and 72 hours. The infected animals secrete mycobacterium bovis in milk and nasal secretions. The disease has significant importance on account of its transmission to humans. *M. bovis* infection is acquired by humans mostly through consumption of un-pasteurized milk contaminated with tuberculous bacilli. In countries where bovine milk is not pasteurized before use, bovine tuberculosis has emerged as the single major cause of extra-pulmonary human tuberculosis (Suliaman *et al.*, 2002).

For identification of *M. bovis* in bovine milk samples, a multiplex PCR was applied containing two sets of primers specific for MTB complex

which amplified at 984 bp (Barouni *et al.*, 2004) and *M. bovis* which amplified at 500 bp (Shah *et al.*, 2002). Out of total 31 animals, nine (29%) showed amplified products at 500 bp specific for *M. bovis* and 11 (35%) were positive for MTB complex. MTB complex primers were used along with *M. bovis* primers to confirm the presence of tuberculosis in animals because there is also possibility of presence of other mycobacterial species of MTB complex than *M. bovis*.

A single PCR by using JB21 and JB22 specific for *M. bovis* also showed incidence of 9(29%) animals secreting *M. bovis* in milk. So 2(6%) positive samples of milk for MTB complex set could be *M. avium* or its related species. It could be detected by using separate primers for *M. avium* or Paratuberculosis which is also common in bovines. The application of single PCR clearly distinguished the bovine TB caused by *M. bovis* from its related causative agents. The three tuberculin positive animals' milk samples were also positive for MTB complex and *M. bovis* (Table I).

A reliable PCR-based diagnostic assay must have a target DNA sequence that is specific for the microorganism to be detected and that must also be present in most, if not all, isolates of the organism. The 500-bp fragment amplified by primers JB21 and JB22 fulfills the first requirement, since it is capable of discriminating *M. bovis* from related strains, such as *M. avium*, which is commonly isolated from cattle, and whose tuberculin is used in the comparative intradermal tuberculin test, and *M. paratuberculosis*, which is also pathogenic to cattle (Bauerfeind *et al.*, 1996). IS6110 has only been detected in species belonging to the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) which present this sequence in multiple copies. In the classical human *M. tuberculosis* variant, the IS6110 element is usually present in 8 to 20 copies. In *M. bovis* strains the IS6110 element is present in two to six copies (Collins *et al.*, 1993; Cousins *et al.*, 1993; Van Soolingen *et al.*, 1994; Van Soolingen *et al.* 1992; Van Soolingen *et al.*, 1991). Only *M. bovis* BCG has a single copy of IS6110, as has been demonstrated in many studies using restriction fragment length polymorphism patterns (Hermans *et al.*, 1990; Szewzyk *et al.*, 1995).

The results presented in this study indicate that the multiprimer method improves tuberculosis diagnosis, with the advantage that this method is rapid and more sensitive than Tuberculin test in animals. PCR could be applied directly to clinical samples in medical and veterinary laboratories, and can be used to detect *M. bovis* in humans and bovines as well.

To conclude PCR for diagnosis of bovine tuberculosis in buffaloes/cattle can be used as a routine diagnostic test for animals' screening or diagnosis in Pakistan.

ACKNOWLEDGEMENT

The financial support of Higher Education Commission, Islamabad is gratefully acknowledged.

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(Received 7 April 2008, revised 13 July 2008)