Detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis* from human sputum samples through multiplex PCR

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Abstract: Tuberculosis (TB) has a long history and being present even before the start of recording history. It has left detrimental effects on all aspect of the life and geared the developments in the science of health. TB is caused by *Mycobacterium tuberculosis* complex (MTBC) including five species *M. tuberculosis, M. bovis, M. africanum*, *M. canetti*, and *M. microti*. *M. tuberculosis* and *M. bovis* infect both animals and humans. Therefore, differentiation of these two closely related species is very important for epidemiological and management purpose. We undertook the present study to characterize mycobacteria isolated from sputum of known TB patients by conventional methods and further, by multiplex PCR (mPCR) to detect the prevalence of Zoonotic TB (TB caused by *M. bovis*). Sputum samples from TB patient were collected from two tertiary care hospitals in Peshawar i.e. Lady Reading Hospital and Hayatabad Medical Complex. All the samples were subjected to Ziehl Neelsen (ZN) stain, culture on Lowenstein Jensen (LJ) and Stone Brink medium, Nitrate reduction test and multiplex PCR. A total of hundred mycobacterial strains were isolated from these samples on the basis of ZN staining, cultural and biochemical methods. Later on, these isolates were subjected to multiplex PCR by using pncATB-1.2 and pncAMT-2 primers specific to *M. tuberculosis* and JB21, JB22 primers specific to *M. bovis*. By means of conventional method, these hundred cultures isolates were differentiated into *M. tuberculosis* (ninety six) and *M. bovis* (four). Furthermore, by mPCR, it was determined that out of hundred isolates, ninety-eight were identified as *M. tuberculosis* and two isolates as *M. bovis*. This molecular method enables to differentiate *M. bovis* from *M. tuberculosis* in human sputum.

Keywords: ZN staining, mPCR, *M. tuberculosis*, *M. bovis.*

INTRODUCTION

In 1882, Robert Koch discovered that TB is caused by *M. tuberculosis*, a small, aerobic nonmotile, bacillus (Dolan *et al*., 2010). TB is a contagious disease and its transmission occurs from person to person through spreading of TB bacilli into the air by an infected person during coughing, sneezing, talking and singing (Beresford and Sadoff, 2010). The MTBC (that causing TB) comprise of five species including *M. tuberculosis, M. bovis, M. africanum, M. canetti* and *M. microti* (van Soolingen, 1997). The most frequent causative agent of TB in humans is *M. tuberculosis*, whilst *M. bovis* is mainly responsible for bovine TB but can also cause TB in humans. In developed countries, zoonotic TB is not a public health problem because of milk pasteurization process (Thoen *et al*., 2006). Approximately 90% of the people infected with TB carry the bacilli for their whole life and may remain unaware of the fact that they are infected with TB (Beresford and Sadoff, 2010).

M. bovis mainly infects cattle but it is an important zoonosis in several countries of the world, which is a serious public health problem and also results in economic losses (Kantor and Ritacco, 1994). The control of bovine TB is difficult because the causative agent, *M. bovis* infects both animals of agricultural importance and wild mammals act as a reservoir (O'Reilly and Daborn, 1995).

The pathogenicity of TB is different in human and cattle. The site of infection in human is usually the apical lobes of the lungs while in cattle, the dorsal caudal regions of the lung are frequently affected by the TB (Cassidy *et al*., 2001). In cattle, the lesion of TB is mostly formed in the lymph nodes associated with the respiratory tract instead of the lung parenchyma (Cassidy, 2006).

M. bovis is transferred to humans, and causes both pulmonary and extra-pulmonary TB. In the dissemination of *M. bovis*, the airborne transmission from human to human is rather controversial as compared to *M. bovis* transmission from cattle to human (LoBue, 2006).

In Nigeria, *M. bovis* was isolated and reported pulmonary TB from patient's sputum specimens (Idigbe *et al*., 1986), from Zaire (Mposhy *et al*., 1983), Latin America (Barrera and Kantor, 1987) and Santa Fe province (Sequeira *et al*., 1990). In Asian countries, small numbers of fig. were related to *M. bovis* infections (Lall, 1969).

The routine PCR techniques used for the detection of MTBC cannot differentiate between *M. tuberculosis* and *M. bovis.* For this reason, it is important that PCR target

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sequence must be precise and specific and it must be present in all the tested isolates. The mtp40 gene (Parra *et al*., 1991) and mpb70 gene (Radford *et al*., 1988) was used earlier to differentiate *M. tuberculosis* from *M. bovis* by PCR protocol. But these methods carry limitations because of the absence of these genes in some strains of *M. tuberculosis* and *M. bovis* (Cousins *et al*., 1991). An 185bp fragment specific to *M. tuberculosis* (De Los Monteros *et al*., 1998) and 500bp fragment specific to *M. bovis* (Rodriguez *et al*., 1995) were identified and characterized. Species-specific identification of *M. tuberculosis* and *M. bovis* was achieved by PCR amplification of the above fragments. The aim of the present study was to characterize mycobacteria isolated from the sputum specimens of known TB patients by conventional methods and molecular identification by mPCR and to detect the prevalence of Zoonotic TB (caused by *M. bovis*).

MATERIALS AND METHODS

A total of One hundred positive sputum specimens were collected from pulmonary TB patients in a wide mouth, transparent containers from the TB wards/chest wards of two tertiary care hospitals named Lady reading hospital and Hayatabad medical complex of Peshawar, Khyber pukhtoonkhwa, Pakistan during 2011. The sputum samples were collected in 50ml sterile plastic bottles and placed in Zip lock plastic bag to avoid leakage of infectious samples. All the samples were subjected to ZN stain, culture on Lowenstein Jensen (LJ) and Stone brink media, Nitrate reduction test and mPCR. The sputum samples with invalid result of above-mentioned parameters were excluded from the study.

Standard digestion-decontamination method (WHO, 1998) was used for processing of sputum samples. In this method, the digestion-decontamination was achieved by three solutions including N-acetyl-L-cysteine (NALC), sodium citrate and sodium hydroxide. NALC-NaOH solution was added in equal amount to sputum samples and left for 15 minutes at room temperature. After room temperature incubation for 15 minutes, phosphate buffer solution was added upto the 50ml mark of the tube and centrifuged for 15 minutes at 3000 g. The supernatant was discarded in antimycobactericidal solution. Then 0.5ml of phosphate buffer was added to the deposit for resuspension. The sediment was inoculated in to four slants: two slants of LJ medium for the growth of *M. tuberculosis* and two slants of Stone brink's medium for the growth of *M. bovis.* A slide was prepared and ZN stain was performed (Quinn *et al*., 1994). The slants were incubated at 37^{o}C for 8 weeks and observed for growth weekly. Slants with positive growth were subjected to Nitrate reduction test for the identification of *M. bovis* and *M. tuberculosis* (Quinn *et al*., 1994). All the chemicals used in sputum processing and media preparation were purchased from Merck, Germany.

For mPCR, the DNA was extracted from culture on LJ media and Stone brink media by using CTAB method (Worden, 2009). In detail, a loopful of colonies from above cultures using a sterile wire loop was inoculated in 800µl of 1X TE buffer, and vortexed thoroughly. Bacteria were heat killed at 85^{o}C for 30 min in a water bath, and then 100µl of 10mg/ml lysozyme was added. Incubated at 37^{o}C for 1 hour in the water bath. A 20µl Proteinase K of (10 mg/ml) and 80µl of 10% sodium dodecyl sulfate was added into the samples and incubated at 65^{o}C for 1 hour in the water bath. Then 100µl of CTAB solution and 100µl of 5M NaCl were added and incubated the samples at 65^{o}C for half an hour. 750µl of cold chloroform isoamyl alcohol (24:1) was added and vortexed for 10 seconds. Then centrifuged at 14,000g for 15 minutes. The aqueous phase containing DNA was transferred to a fresh autoclaved eppendorf tube and added 450µl of isopropanol. Then slowly moved the tubes upside down for DNA fibers to appear. Kept the samples at -20^{o}C for overnight precipitation, and then centrifuged the tubes at 14,000 g for 15 minutes. The supernatant was discarded carefully and ice cooled 70% ethanol was added to the samples. The tube was slowly moved upside down to wash the pellet and again centrifuged as above. The pellet was dried in the vacuum dryer for 3-5 minutes. The pellet was reconstituted in 50-100µl 1X TE buffer and the DNA samples were stored at 4^{o}C for PCR, also the DNA was quantified by taking their optical density. For long term storage, DNA samples were kept at -70^{o}C.

Five microlitres of the extracted DNA was subjected to (0.8%) agarose gel electrophoresis for determination of the quality (Santha *et al*., 2005). The extracted DNA from the cultures was subjected to mPCR by using two sets of primers: pncATB-1.2 (ATGCGGGCGTTGATCATC GTC), pncAMT-2 (CGGTGTGCCGGAGAAGCGG) for *M. tuberculosis* and JB21 (TCGTCCGCTGATGCAAGT GC), JB22 (CGTCCGCTGACCTCAAGAAG) for *M. bovis* (Dawit *et al*., 2002) (table 1).

Amplification was done in a 50µl reaction mixture which contained 4µl of PCR buffer; 5µl of $MgCl_2$; 5µl of 200-mM dATP, dGTP, dCTP, and dTTP; 0.5µl Taq polymerase, 1.6µl each of both primers Forward (F) and Reverse (R), and 5µl genomic DNA solution. The PCR conditions set as, initial denaturation done at 95^{o}C (1 minute), denaturation consisted of 30 PCR cycles at 94^{o}C (1 minute) annealing at 67^{o}C (1 minute), extension at 72^{o}C (1 minute), and final elongation at 72^{o}C (10 minutes). All the chemicals and reagents used in DNA extraction and PCR reactions were purchased from Fermentas, Germany.

All the PCR reactions were carried out in an automated thermocycler (Biorad CFX96). After amplification, the PCR mixture was analyzed by the 1.5% agarose gel electrophoresis. For each mPCR test distilled water was

Table 1: Primer name, target and its sequence

Primer	Target	Sequence (5 -3)
pncATB-1.2 (forward)	pncA gene	TGCGGGCGTTGATCATCGTC
pncAMT-2 (reverse)	pncA gene	CGGTGTGCCGGAGAAGCGG
JB21 (forward)	hupB gene	TCGTCCGCTGATGCAAGTGC
JB22 (reverse)	hupB gene	GTCCGCTGACCTCAAGAAAG

Table 2: Results of ZN stain, culture, nitrate reduction and multiplex PCR of sputum samples for MTBC

Total samples (n)	ZN positive n (%)*	Growth on LJ media and Stone brink media n (%)*	Nitrate reduction test n (%)*	Multiplex PCR detection	
				M. tuberculosis n (%)*	*M. bovis* n (%)*
100	100 (100)	96 (96)	96 (96)[A] and 4 (4)[B]	98 (98) [C]	2 (2)[C]

Key: n (%)*= number (percentage) A = Positive Nitrate reduction test B = Negative Nitrate reduction test C = Total PCR positive result

used as negative control and $H_{37}Rv$ was used as positive control.

RESULTS

One hundred sputum specimens were collected from patients with active pulmonary TB. Out of these hundred patients representing all ages groups, thirty five were males and sixty five were females, belonging to the Pushtoon tribes living in the city and remote areas of Peshawar, Pakistan.

ZN staining of direct sputum samples revealed, that all of them were smear positive. All the isolates were grown on either LJ medium or Stone brink medium (fig. 1).

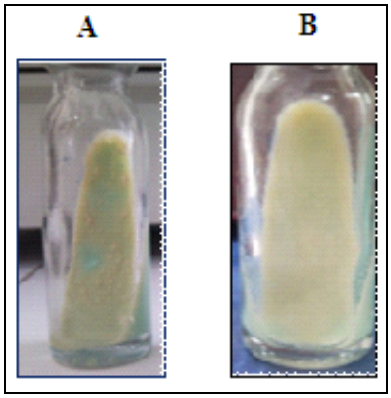

Fig. 1: Growth of *M. tuberculosis* on LJ medium (A) and *M. bovis* on stone brink medium (B).

Out of hundred isolates ninety-six were identified as *M. tuberculosis* and four as *M. bovis* on the basis of their growth on respective medium, and nitrate reduction test (fig. 2).

All the mycobacterial isolates were subjected to mPCR using *M. tuberculosis* specific primers pncATB-1.2 and pncAMT2 and *M. bovis* specific primers JB21 and JB22 for their differential identification (table 2).

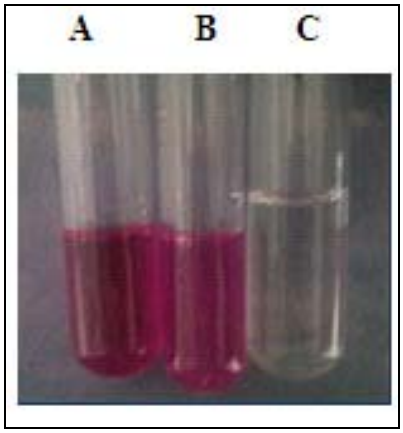

Fig. 2: Nitrate reduction by *M. tuberculosis* (B), Positive control (A) and negative control (C) were shown.

PCR amplification yielded two types of amplicon, a 500bp long for two of the four *M. bovis* isolates that were identified by conventional method, while ninety eight strains generated the amplification product of 185bp along with reference strain of *M. tuberculosis* $H_{37}Rv$ (figs 3 and fig. 4).

DISCUSSION

PCR is a quick and sensitive technique for the isolation and identification of MTBC from different clinical specimens in TB laboratories (Clarridge *et al*., 1993). It is very difficult to diagnose the infections by PCR method and it is also problematic to declare whether this infection is caused either by *M. bovis* or *M. tuberculosis.* Due to the absence of mtp40 gene in some strains of *M. tuberculosis*, it is very difficult to develop species-specific PCR method to distinguished *M. tuberculosis* from *M. bovis* and these techniques are now abundant because of the false negative results (Weil *et al*., 1996). In this research, mPCR was developed for 500bp *M. bovis* specific fragment (Rodriguez *et al*., 1995) and 185bp *M. tuberculosis* specific fragment (De Los Monteros *et al*., 1998) based

on one step amplification. Using DNA from *M. tuberculosis* and *M. bovis*, an amplified product of 185bp and 500bp was isolated respectively.

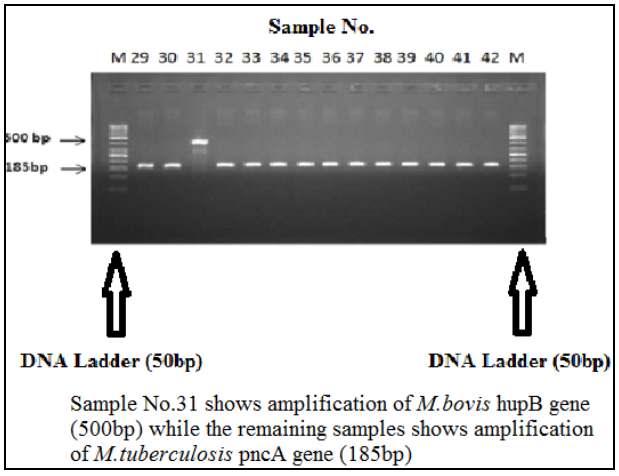

Fig. 3: Amplification of *M. tuberculosis* pncA gene (185bp) and *M. bovis* hupB gene.

Out of hundred isolates, ninety-eight were found *M. tuberculosis* while two were identified as *M. bovis* by mPCR. It has been confirmed that species-specific 500bp product is present in *M. bovis* as reported earlier (Weil *et al*., 1996). Our study revealed that *M. bovis* account for 2% cases of pulmonary TB, which is very close to the previously reported percentage (2.1%) in literature by Gervois *et al* (1972). In Egypt, two labs analyzed the positive TB sputum samples for the detection of *M. bovis* at three occasions and it showed three different results i.e. 0.4%, 6.4% and 5.4% (Elasabban *et al*., 1992) whilst our determined percentage (2%) falls between these percentages. In Nigeria, 102 *M. tuberculosis* complex were isolated from sputum specimens of active TB patients, 4 of them were *M. bovis* (Idigbe *et al*., 1986), which is slightly high then repeated in our work. Another study in Nigeria also reported 10% prevalence of *M. bovis* in pulmonary TB, which is an alarming proportion (Idrisu and Schnurrenberger, 1977). Nawaz *et al*. (2012) reported 5% *M. bovis* in a study conducted in Lahore, Pakistan which is also close to our finding.

The mPCR assay described in the present study is based on amplification occurring in one tube and identification of a 500bp *M. bovis* specific product and 185bp *M. tuberculosis* specific product; it has also been established by several other researchers for the detection of *M. tuberculosis* and *M. bovis* (Shah *et al*., 2004). Thus, 500bp and 185bp specific fragments could be of potential value in differential identification of *M. bovis* and *M. tuberculosis* isolates, which is otherwise difficult with the conventional procedures.

Some researchers have recently reported the existence of 500bp *M. bovis* specific fragment in classical strains of *M. tuberculosis* (Prabhakar *et al*., 2004); however in the present study none of the ninety-eight *M. tuberculosis* strains of human origin tested showed the presence of this element.

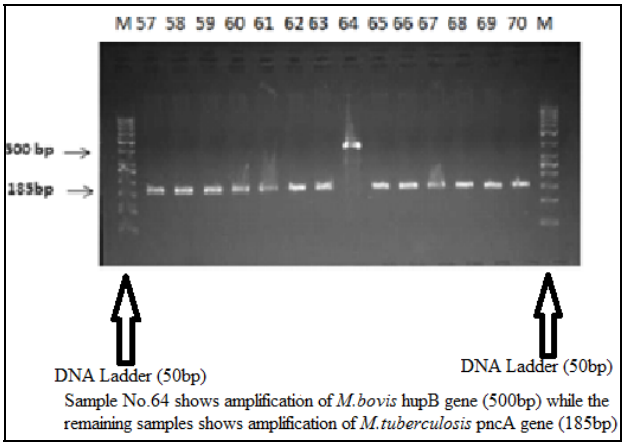

Fig. 4: Amplification of *M. tuberculosis* pncA gene (185bp) and *M. bovis* hupB gene (500bp).

The PCR conditions consisted of the following parameters: initial denaturations were 95^{o}C (10 min), followed by denaturation having 30 PCR cycles at 94^{o}C (1 min), annealing at 67^{o}C (1 min), and extension at 72^{o}C (1 min) and a final extension of 72^{o}C (10min); these conditions are in line with previous study (Shah *et al*., 2004).

In the present observations, JB primer set targets the hup B gene locus, while pncA primer set target were directed against *M. tuberculosis* in the pncA gene, and as a result the desired size fragments were amplified, as previously reported (Dawit *et al*., 2002). The same primers were also used by Nawaz *et al*. (2012) and Lilian *et al*. (2014) and as a result both 185bp and 500bp desire size fragment were amplified.

In conclusion, the mPCR used in this study has the ability to segregate *M. bovis* from *M. tuberculosis*, as a single product was obtained in all the cases, 185bp for *M. tuberculosis* and 500bp product for *M. bovis*.

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