Research Article

Rapid and specific detection of *Mycobacterium tuberculosis* directly from sputum specimens using IS6110 and *pncA* through multiplex-PCR

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Abstract

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* complex, is the second leading cause of death. One third of the world's population is infected with TB. Every day more than 23000 people develop active TB and nearly 5000 die. Approximately 20-50% of patients with TB are smear negative, accounting for as much as 17% of TB transmission, posing an important public health hazard. In the objective of improved TB diagnosis and rapid detection of *M. tuberculosis* complex species particularly from paucibacillary sputum specimens, the sensitivity of Polymerase Chain Reaction (PCR) using IS6110 and *pncA* as genetic targets was compared with the conventional fluorescent microscopy. The study also aimed to check the sensitivity of two different genetic markers IS6110 and *pncA* for the detection of *M. tuberculosis*. Sputum specimens were collected from TB suspected subjects (N= 200; males=108; females=92; mean age=47±18.4) attending outpatient department at Fatima Jinnah General and Chest Hospital, Quetta. All the samples were decontaminated using N-acetyl-L-cysteine (NALC)-NaOH method and were subjected to auramine-O fluorescent microscopy and multiplex PCR using TB1 and TB2 primers specific for *M. tuberculosis* complex and *pncATB*-1.2 and *pncAMT*-2 primers specific to *M. tuberculosis*. Out of 200 specimens, *M. tuberculosis* around 15 (7.5%) was detected by fluorescent microscopy and 33 (16.5%) were detected through

PCR, respectively. Whereas 18 smear negative specimens were found to be positive by PCR. Statistically significant difference between fluorescent microscopy and PCR was observed ($p < 0.001$). Among these 33 positive cases, 18 (16.6%) were males and 15 (16.30%) were females with statistically no significant difference (OR: 1.03, 95% CI: 0.48-2.17; $p > 0.05$). Similarly, age was not found to be associated ($p > 0.05$) with the increased risk of TB, however, the disease incidence was more prevalent (20.89%) in patients aged 41–60 years. Multiplex PCR showed increased sensitivity as compared to fluorescent microscopy for TB diagnosis and will be a useful tool in detecting smears negative TB. Further, it can also be concluded that both genetic markers *IS6110* and *pncA* showed similar sensitivity in the detection of *M. tuberculosis*.

Keywords: Quetta; Tuberculosis; Paucibacillary; Diagnosis; Genetic markers

Introduction

Tuberculosis is an ancient, extensively prevalent, and more often deadly, contagious disease caused by *M. tuberculosis* complex species, usually *M. tuberculosis* [1]. TB has proceeded to be a “slate wiper” in the human history throughout centuries and was titled “consumption” and “white plague” in the 20th century. It was world’s major cause of death from all causes, responsible for one death in every seven cases [2]. Today’s statistics are still alarming. Globally, TB ranks 2nd major cause of death after HIV by an infectious disease and WHO estimates that some 2 billion individuals, 1/3rd population of the world, are infected with *M. tuberculosis* [3]. Each day some 23000 new TB cases develop and nearly 5000 people die. Annually, approximately 8.7 million active TB cases and 1.7 million deaths are reported. If TB was not massively controlled by TB control programs, another estimated 1 billion people globally will become infected and 40 million will die of TB by 2020. Mortality is highest in developing countries, where over three-quarters of TB-cases occur [2, 4].

TB is prevalent and a serious national health problem in Pakistan. Globally, Pakistan ranks 5th among the 22 High Burden Countries (HBCs) and 4th among the 27 countries with highest burden of Multi Drug Resistant (MDR) TB [3]. About 420,000 new cases of TB develop every year, with an incidence of 231/100,000 and 60,000 people

die of the disease. Of these (420,000), around 175,000 cases are sputum smear positive and 9000 are the cases of MDR TB while remaining of the cases are “sputum smear negative TB” [5].

The most common technique for diagnosis of TB is direct smear microscopy [3]. The smear microscopy is rapid and an inexpensive method. However, it lacks sensitivity and specificity particularly with smear negative cases [6]. Bacterial culture is the gold standard; however, culture usually take six to eight weeks due to the slow growth of *M. tuberculosis* on solid media, delaying time to results, followed by identification with use of biochemical or molecular methods [7].

Early and rapid diagnosis is of major importance in instituting effective, timely therapy and for the control of TB [7]. A fast and reliable diagnosis would greatly improve the control of the TB [8]. The need for rapid diagnosis has led to the development of PCR for direct detection of mycobacterial DNA in clinical specimens [9]. Some of the targets used to amplify the DNA of *M. tuberculosis* include 65kDa, 38kDa antigen coding regions and insertion sequence *IS6110*. Most studies have generally targeted the *IS6110* sequence [10, 11]. *IS6110* is a repeated DNA sequence found in all *M. tuberculosis* complex genomes described by Eisenach *et al.* [12] and used for the diagnosis TB. Since then, *IS6110*-PCR has been routinely used [13]. The *pncA* gene, which encodes the

pyrazinamidase enzyme (PZAase) that converts the pro drug pyrazinamide (PZA) to pyrazinoic acid (POA), has a single point mutation at nucleotide 169 G > C (appears to be unique to *M. tuberculosis*) is one of the markers that is commonly used to distinguish *M. tuberculosis* from others MTC species [14, 15]. In the current study we investigated to detect *M. tuberculosis* from paucibacillary sputum specimens by simultaneous one step amplification of IS6110 present in *M. tuberculosis* complex species and *pncA* specific for *M. tuberculosis*.

Materials and methods

Area of study

Quetta is the provincial capital of Balochistan and 6th largest city of Pakistan with 2.8 million populations. The population belongs to different ethnic groups. Collection of sputum specimens, decontamination and fluorescent microscopy was performed at Provincial Reference Laboratory (PRL) for TB at Fatima Jinnah General and Chest Hospital and PCR was performed at Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta.

Collection of the sputum specimens

A total of 200 sputum samples (male=102 and female=92) were collected in sterile pre-labeled containers. Specimens were collected from all TB suspects showing the classical symptoms of disease such as the history of prolonged more than two weeks' chronic cough, fever and chest pain. Gender and age of the TB-suspects were also recorded.

Sample processing

All the sputum specimens were decontaminated and liquefied using the standard *N*-Acetyl-L-cysteine (NALC)-NaOH method [16] and concentrated by centrifugation in Biosafety Level III Provincial Reference Laboratory for TB.

The resuspended sediments were used for preparation of AFB smears and rest (500 µl) of the sediments were rendered non-infectious by heat killing. The heat killed samples were then transported to CASVAB and stored at -20°C until used for PCR.

Fluorescent microscopy

Smears were stained with auramine-O staining technique [16]. All the smears were then examined under florescent microscope using 40X objective.

Polymerase chain reaction

DNA was extracted using standardized cetyltrimethyl ammonium bromide (CTAB) method as described by Ausubel *et al.* [17]. Extracted DNA was subjected to PCR using two sets of oligonucleotide primers (Macrogen) as shown in Table 1. The multiplex PCR was carried out by amplifying 123-bp fragment of the IS6110 specific for *M. tuberculosis* complex and 185-bp fragment of the *pncA* gene for specific for *M. tuberculosis* (Figure 1).

The multiplex PCR was performed in a total volume of 25 µl reaction mix which contained 1X Taq buffer, 2.5mM of MgCl₂, 50µM of each dNTP, 1.25 U of Taq DNA polymerase, 10 pmol of each of both oligonucleotide primers IS6110 and *pncA* (Forward and Reverse), and 4µl Template DNA. Amplification was performed using automated thermal cycler (Applied Biosystems, 2720). The mixture was first denatured at 96°C for 5minutes, followed by 35 cycles of PCR with denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, extension at 72°C for 45 seconds and the final extension at 72°C for 7 minutes. A negative control (without DNA template) and a positive control containing *M. tuberculosis* DNA were also included in each PCR run. The amplified PCR products were analyzed by agarose gel electrophoresis (2% W/V) at 100 Volts for 1 hour followed by staining with ethidium bromide (0.5mg/ml). The electrophoresed

amplicons were visualized and documented using UV transilluminator (Wealtec Dolphin-View, USA). The 50-bp DNA

marker (Vivantis, Malaysia) was used as a reference to estimate the DNA bands.

Table 1. Sequence of oligonucleotide-primers used for amplification of *IS6110* and *pncA* gene fragments and the size of amplicons produced

Primer	Primer sequence (5'-3')	Target	Amplicon Size
TB1(F)	CCTGCGAGCGTAGGCGTCGG	<i>IS6110</i>	123-bp
TB2 (R)	CTCGTCCAGCGCCGCTTCGG		
<i>pnc</i> ATB-1.2 (F)	ATG CGGGCGTTGATCATCGTC	<i>pncA</i>	185-bp
<i>pnc</i> AMT-2 (R)	CGGTGTGCCGGAGAAGCGG

Figure 1. *IS6110* and *pncA* multiplex-PCR for the detection of *M. tuberculosis* complex and specific identification of *M. tuberculosis* directly from clinical sputum specimens

Legend. PC: Positive Control. NC: Negative Control. Lane 1, 3, 6, 7, 8, 9, 10, 12, 13, 14 and 15: Positive for *M. Tuberculosis* complex and *M. tuberculosis*. Lane 2, 4, 5, 11 and 16: No amplification: Negative for *M. tuberculosis* complex and *M. tuberculosis*

Results

Out of 200 sputum specimens, 92 (46.7%) were females and 108 (53.3%) were males. The sputum specimens were analyzed for the detection of *M. tuberculosis* through the conventional fluorescent microscopy and the PCR targeting *IS6110* and *pncA*. In the current study, 15 (7.5%) sputum specimens were positive by fluorescent microscopy and 33 (16.5%) by PCR with both genetic markers (Figure 2). All the 15 smear positive specimens were also positive by PCR. Among these 33 (16.5%) positive samples 18 (16.6%) were males and 15 (16.30%) were females. The sensitivities of auramine-O fluorescent microscopy, *IS6110* and *pncA*-PCR were 45%, 100% and 100%,

respectively, whereas the specificities of all three were 100% (Table 2).

Sputum sample analysis revealed that both males and females were approximately at equal risk of TB infection (OR= 1.03, 95% CI: 0.48–2.17). Therefore, the difference in the incidence of TB between males and females was statistically non-significant ($\chi^2=0.005$, df= 1, $p=0.945$). The proportions of study subject with age categorized as 1–20, 21–40, 41–60 and >60 were observed 15%, 33.0%, 33.55%, and 18.50%, respectively. TB patients with age 41–60 years were 1.7-fold more likely to be infected with TB as compared with patients less than 20 years of age (95% CI: 0.25–2.45). However, age was

not found to be statistically associated with TB risk ($\chi^2 = 1.33$, $df=3$, $p= 0.722$) (Table 3).

Figure 2. Bar chart showing the frequency of positive TB cases detected through fluorescent microscopy and PCR using *IS6110* and *pncA* specific genetic markers.

Table 2. Distribution of tubercle bacilli detected through fluorescence microscopy and PCR test obtained from clinically suspected TB patients, Quetta (n=200)

Result for specimen= 200	Fluorescent microscopy		IS6110-PCR		pncA-PCR	
	+	–	+	–	+	–
Positive TB cases	15	18	33	0	33	0
Negative TB cases	0	167	0	167	0	167
Sensitivity (%)	45.45%		100%		100%	
Specificity (%)	100%		100%		100%	

Table 3. Gender and age-wise distribution of TB among clinically suspected subjects in Quetta, Pakistan, 2016 (N=200)

Variable	Total no. of Specimens N (%)	<i>M. tuberculosis</i>			OR (95% CI)	P value
		Positive cases N (%)		Negative N (%)		
		Smear +ve	PCR +ve			
Gender						0.945 ^{ns}
Female	92 (46.0)	6 (6.5)	15 (16.3)	77 (83.7)	Ref (1.0)	
Male	108 (54.0)	9 (8.3)	18 (16.7)	90 (83.3)	1.03(0.5–2.2)	
Age						0.722 ^{ns}
01–20	30 (15.0)	3 (10.0)	4 (13.3)	26 (86.7)	Ref (1.0)	
21–40	66 (33.0)	5 (7.6)	11 (16.7)	55 (83.3)	1.3 (0.2–4.2)	
41–60	67 (33.5)	4 (6.0)	14 (20.9)	53 (79.1)	1.7 (0.2–2.4)	
>60	37 (18.5)	3 (8.1)	5 (13.5)	32 (86.5)	1.0 (0.2–1.8)	

P^{ns}> 0.05 (non-significant); OR: Odds Ratio; CI: Confidential Intervals; Ref: Reference

Discussion

The TB diagnosis is a major problem and a serious challenge, especially in developing countries such as Pakistan. The early

diagnosis of TB particularly with the paucibacillary specimens such smear negative specimens poses a great challenge [18]. In Pakistan, smear microscopy is the

sole laboratory method for the TB diagnosis in addition to radiography. There are no appropriate diagnostic facilities in most of the hospitals in Balochistan. The delay in diagnosis and treatment is one of the major obstacles in the control of TB [19]. Microscopy is a fast, inexpensive and rapid diagnostic method. However, microscopy lacks sensitivity and specificity. It can only detect 60 to 70 % acid-fast bacilli in culture positive specimens [20] and it also fails to differentiate between dead and viable bacteria [21, 22]. The culture technique is although a “gold standard” but is time consuming.

Early diagnosis of TB is necessary to disrupt the disease transmission chain. Smear positive patients are considered highly infectious and being focused by most of the clinicians. However, smear negative patients are also reported to be responsible for approximately 17% of transmission and its impact on public health could not be neglected [23]. Smear negative cases are the potential threats and contribute to the transmission of disease. Therefore, the current study was designed to rapidly diagnose smear negative cases by PCR.

We compared the conventional microscopy and PCR for the rapid diagnosis of TB. An important utility of PCR is its potential for the detection of TB from specimens with low bacterial load. 15 (7.5%) out of 200 cases were smear-positive and 33 (16.5%) were PCR- positive with both genetic targets, indicating the fact that PCR is more sensitive and reliable diagnostic technique. All 15 smear-positive sputum specimens were positive by PCR for *M. tuberculosis*. PCR could also detect 18 smear negative specimens. multiplex PCR was performed using oligonucleotides for IS6110 and *pncA* gene amplification. Both genetic targets were equally sensitive and specific. This molecular method enables to differentiate *M. tuberculosis* from other *M. tuberculosis*

complex species. The sensitivity of smear-microscopy and PCR was 45.45% and 100 respectively.

The higher rate of diagnosis by PCR compared with smear microscopy is also evidenced by other studies [24-26]. The higher positivity of PCR is attributed to the fact that fewer than ten mycobacteria could be detected and the repetitive nature of the target sequence IS6110 amplified by PCR. The comparative result of two techniques used for TB diagnosis indicated a statistically significant difference ($\chi^2=82.064$, df= 1, $p<0.001$).

Most PCR assays amplify fragments from *M. tuberculosis* complex and thus cannot distinguish between infections caused by *M. tuberculosis* and other *M. tuberculosis* complex species. Routine application of PCR-based methods requires that the target sequence should be highly specific. The insertion sequence “IS6110” which exists only in *M. tuberculosis* complex species, is the most used target for the mycobacterial DNA amplification [27-29] and is the standard marker for the epidemiologic studies of TB. Moreover, the “IS6110” is a potential tool to differentiate between the members of *M. tuberculosis* complex and other non-tuberculosis and atypical mycobacteria [30, 31]. The *pncA* gene containing single nucleotide polymorphism (a cytosine residue at position 169) is specific for *M. tuberculosis* [32] and is a potential genetic marker used to differentiate *M. tuberculosis* from other *M. tuberculosis* complex species. The *pncA* gene containing specific mutations was found to be involved in pyrazinamide sensitivity.

The current study revealed that sex was not associated with increased risk of TB infection ($p>0.05$). However, positive TB cases were detected slightly more in males than females (18 versus 15 cases). Several other studies reported by Ndungu *et al.* [33] in Kenya and Fleming *et al.* [34] in Russia

cited the males as being more likely to be infected with TB infection. WHO report on TB incidence and gender demonstrated that prevalence of TB infection was higher in males at all ages except in childhood and that the gender differential incidence appears to begin at age ten and sixteen years; reasons for increased incidences and prevalence among males are poorly understood and need further investigation [35].

Age is implicated as one of the important risk factors for TB. Patients aged between 41–60 years were 1.7-fold (95% CI: 0.25 – 2.45) at higher risk of acquiring the disease compared with <20 years old patients (20.89% vs. 13.0%). This may be attributed to weakened and compromised immune system in the old age. However, the age was not found significantly associated with TB infection ($p>0.05$). This finding is consistent with the previous studies which cited TB infection to be considerably increased with age [36–38].

Conclusion

In conclusion, the findings of our study strongly suggested the use the multiplex PCR using the “IS6110” and “pncA” in the routine analysis as a potential tool for the rapid TB diagnosis due to its increased sensitivity.

Author’s contributions

Conceived and designed the experiments: MA Awan, M Rizwan & M Shafee, Performed the experiments: M Rizwan, Analyzed the data: M Naeem, SK Achakzai, S Ashraf & Z Ahmed, Contributed reagents/ materials/ analysis tools: A Samad. Wrote the paper: H Haider & M Rizwan & FS Bugti.

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