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Detection of rifampicin resistance of *Mycobacterium tuberculosis* using multiplex allele specific polymerase chain reaction (MAS-PCR) in Pakistan

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ABSTRACT

Drug resistance in tuberculosis (TB) is a major public health challenge in developing countries such as Pakistan. Multiplex allele specific polymerase chain reaction (MAS-PCR) is a DNA amplification method that could contribute to rapid detection and control of drug resistant tuberculosis (DR-TB) in Pakistan. The purpose of this study was to test the utility of MAS-PCR to detect resistance in Pakistan. Drug susceptibility testing (DST) was used to identify rifampicin resistant and susceptible clinical isolates from TB cases in Pakistan. MAS-PCR was used to detect the most frequent mutations in the gene *rpo*B among 213 resistant and 37 susceptible isolates. Among 213 clinical isolates, MAS-PCR identified mutation D435Y (Asp435Tyr) in 24 (11.3%) cases, H445Y (His445Tyr) in 14 (6.6%), S450L (Ser450Leu) in 124 (58.2%) and S450W (Ser450Trp) in 18 (8.4%) cases. MAS-PCR did not detect known mutations in 33 (15.5%) cases. Among 12 cases, a novel mutation at codon 434 (Met434Ile) and a common variant at codon 435 (Asp435Tyr) was detected in *rpo*B gene which is indicative of double mutation. In 4 isolates, a novel mutation at codon 432 (Gln432Pro) was identified. In an additional 4 isolates, mutations Met434Val and His445Asn were identified. Moreover, a mutation in *rpo*B (Leu452Pro) was found in 5 isolates. DNA sequencing confirmed the absence of mutations in *rpo*B in the 8 remaining isolates. MAS-PCR had 88.3% sensitivity and 100% specificity using DST as the reference, which suggested that this method could be implemented as an initial marker for screening of multi-drug resistant tuberculosis (MDR-TB) in Pakistan.

1. Introduction

Multi-drug resistant tuberculosis (MDR-TB) is a term used for *Mycobacterium tuberculosis* (MTB) strains resistant to rifampicin (RIF) and isoniazid (INH). Globally in 2016, 4.1% of newly diagnosed TB cases and 19% of previously treated cases were estimated to have MDR-TB (World Health Organization, 2017). In Pakistan, the proportion of MDR-TB in newly diagnosed cases is 4.3% and in previously treated cases is 19.4% (Javaid et al., 2016; Ullah et al., 2016a). Point mutations in certain genes identified by MAS-PCR correlate with phenotypic MTB drug resistance (Imperiale et al., 2011). Single base nucleotide mutations either by insertion, deletion or via substitution in the rifampicin resistance determining region (RRDR) affect rifampicin (RIF) binding by decreasing the affinity to RNA polymerase of MTB (Cole et al., 1998; Rastogi and David, 1993). About 95–97% of mutations occur in the RRDR in rifampicin resistant isolates. These mutations are located in codons 426 to 452 (Chia et al., 2012; Ullah et al., 2016b); specifically, they occur in codons 430, 432, 435, 441, 445, 450, and 452 (Chan et al., 2007; Yip et al., 2012).

As MDR-TB is difficult to treat, rapid detection methods are required

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to avoid incorrect treatment and to prevent transmission. The "gold standard" drug-susceptibility testing (DST) is a slow process due to slow growth of MTB. DST is not routinely carried out in resource limited settings like Pakistan due to cost, required laboratory infrastructure and technical expertise. Nonetheless, MAS-PCR is a rapid, simple and inexpensive technique used for the detection of MDR-TB (Yang et al., 2005; Allegui et al., 2012a). Therefore, we assessed the ability of MAS-PCR to detect the most common mutations in codons 435, 445 and 450 and compared it with DST and DNA sequencing.

2. Material and methods

2.1. Study setting and population

This study was carried out at the Department of Bacteriology, Institute of Public Health, Lahore, Punjab Province, Pakistan. It is the largest TB unit in the province covering more than 30 TB endemic districts. A total of 2445 archived clinical isolates were collected during the period January 2012 to December 2013. Non-tuberculous mycobacteria (NTM) and those isolates on which DST could not be applied were excluded from the final analysis.

2.2. Laboratory analysis

Para-nitro benzoic acid test and Bactec Nap TB differentiation test (Becton Dickinson, Sparks, MD, USA) were used to confirm that the colonies were from the MTB complex (Hasan et al., 2010). DST was performed on Löwenstein-Jenson media using rifampicin (40 µg/ml) and 1% proportion method as per standard guidelines (India T, 2009). DNA extraction from MTB cultures was performed using the standard cetyltrimetyl ammonium bromide method (van Soolingen et al., 2001).

2.3. MAS-PCR

All RIF resistant and 37 randomly selected susceptible isolates by DST were subjected to MAS-PCR to detect mutations in the codons 435, 445 and 450 of the *rpo*B gene using purified DNA from cultured cells (Details in supplementary materials). Primers were designed using Primer3 software to amplify the region of interest (Table 1). These codons were selected because they are the most frequently involved in RIF drug resistance (Kourout et al., 2009; Evans et al., 2009).

2.4. DNA sequencing

The RRDR of *rpo*B gene was sequenced to identify mutations in RIF-resistant isolates where MAS-PCR did not detect resistance. Cycle sequencing was performed using dideoxy chain termination chemistry. Amplified products were sequenced in sense and antisense direction using ABI310 genetic analyzer (Life technologies, USA) (Details in supplementary materials). Sequenced data was analyzed using Bio Edit sequence alignment software.

2.5. Statistical analysis

Statistical analysis was performed using Microsoft Excel. Diagnostic efficacy of MAS-PCR was measured in terms of specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) (Altman et al., 2013).

3. Results

Out of total 2445 isolates, 47 were found to be NTM. In total, 2367 isolates were included after excluding 31 cases on which DST could not be applied.

3.1. Correlation between DST and MAS-PCR results

Among 2367 isolates, 213 were found resistant to RIF as determined by DST. MAS-PCR of 213 RIF resistant isolates showed mutation D435Y (Asp435Tyr) in 24 cases (11.3%) (Fig. 1), H445Y (His445Tyr) in 14 (6.6%) (Fig. 2), S450L (Ser450Leu) in 124 (58.2%) and S450W (Ser450Trp) in 18 (8.4%) (Fig. 3 & Table 2). MAS- PCR did not detect known mutations in 33 (15.5%) cases (Table 2). MAS-PCR showed no mutation in 37 susceptible isolates as determined by DST. MAS-PCR had a sensitivity of 88.3% with 100% specificity based on DST as reference. PPV and NPV were 100% and 52.9%, respectively.

3.2. DNA sequencing

Thirty-three RIF resistant isolates that were not detected by MAS-PCR were bi-directionally sequenced. Among 12 cases, a novel mutation at codon 434 (Met434Ile) and a common variant at codon 435 (Asp435Tyr) was detected in *rpo*B gene which is indicative of double mutation. In 4 specimens, a novel mutation at codon 432 (Gln432Pro) was identified while in an additional 4 specimens, Met434Val and His445Asn were identified. Moreover, a mutation in *rpo*B gene (Leu452Pro) was detected among 5 of the isolates. No mutations were detected among the 8 remaining RIF-resistant isolates.

4. Discussion

In this study, MAS-PCR showed excellent performance, with 88.3% sensitivity and 100% specificity, similar to previous studies (Allegui et al., 2012b; Gupta et al., 2013). The sensitivity of MAS-PCR in our study was higher than reported in one other study (Molina-Moya et al., 2015) and lower than reported elsewhere (Imperiale et al., 2011; Chia et al., 2012; Vadwai et al., 2012).

The most common mutations were found in *rpo*B codons S450 L and S450 W (58.2 & 8.4%), consistent with other studies in Brazil (60 & 0%) (Höfling et al., 2005), Russia (57 & 3.8%) (Afanas' ev et al., 2007) and Pakistan (54.5 & 9.1%) (Khan et al., 2013). However, it was higher than reported previously in China (27.7 & 9.2%) (Yue et al., 2003) and Mexico (41.7% & 0%) (Ramaswamy et al., 2004), and lower than that in Georgia (77.8 & 0%) (Gegia et al., 2008) and Cape Town (83 & 0%) (Van Rie et al., 2001). The mutation at codon 450 (TCG > TTG)

Table 1
Primers used for MAS-PCR to detect specific codon of *rpo*B gene.

Primer Name	Sequence	Codon # in *Escherichia coli* (*E.coli*)	Codon # in MTB	Product Size
*rpo*B-F1	5′-TCGCCGCGATCAAGGAGTTC-3′	External	External	249
*rpo*B-R	5′-TGACCCGCGCGTACACCGAC-3′			
*rpo*B-F2	5′-GCTGAGCCAATTCATGGACCA-3′	516	435	215
*rpo*B-R	5′-TGACCCGCGCGTACACCGAC-3′			
*rpo*B-F3	5′-GTCGGGGTTGACCCACA-3′	526	445	182
*rpo*B-R	5′-TGACCCGCGCGTACACCGAC-3′			
*rpo*B-F4	5′-ACAAGCGCCGACTGTCGGCG-3′	531	450	168
*rpo*B-R	5′-TGACCCGCGCGTACACCGAC-3′			

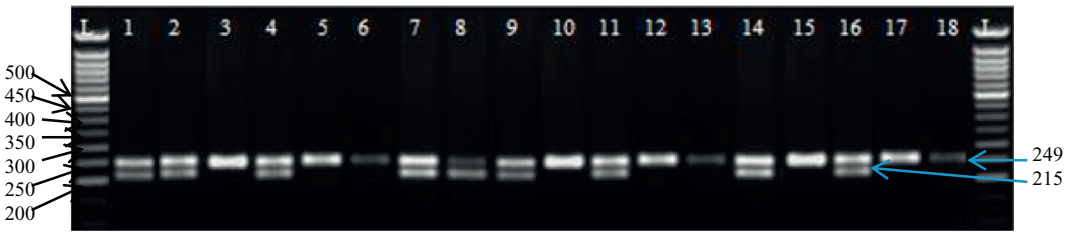

Fig. 1. Agarose gel electrophoresis pattern of PCR amplified product for MAS-PCR assay of *rpo*B codon 516 mutation analysis, Ladder on both side; Size standard 50 bps, Lane 1; H37Rv positive control, Lane 2, 4, 7, 8, 9, 11, 14, 16; strains with *rpo*B codon 516 susceptible alleles, Lane 3, 5, 6, 10, 12, 13, 15, 17, 18; strains with *rpo*B codon 516 mutant alleles.

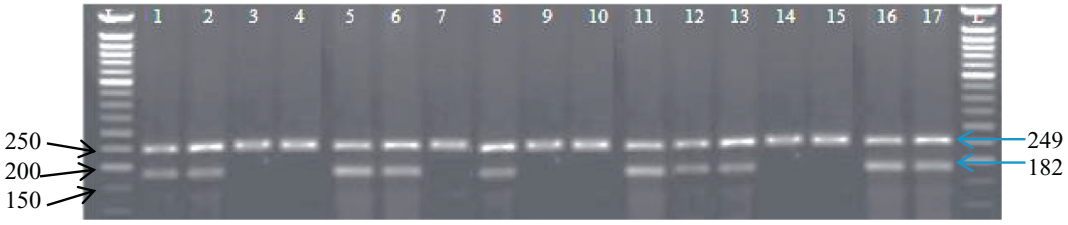

Fig. 2. Agarose gel electrophoresis pattern of PCR amplified product for MAS-PCR assay of *rpo*B codon 526 mutation analysis, Ladder on both side; Size standard 50 bps, Lane 2, 5, 6, 8, 11, 12, 13, 16, 17; strains with *rpo*B codon 526 susceptible alleles, Lane 3, 4, 7, 9, 10, 14, 15; strains with *rpo*B codon 526 mutant alleles, Lane 1; H37Rv positive control.

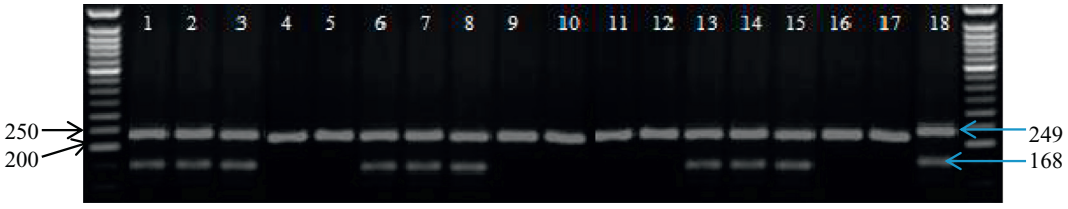

Fig. 3. Agarose gel electrophoresis pattern of PCR amplified product for MAS-PCR assay of *rpo*B codon 531 mutation analysis, Ladder on both side; Size standard 50 bps, Lane 1; H37Rv positive control, Lane 4, 5, 9, 10, 11, 12, 16, 17; strains with *rpo*B codon 531 mutant alleles, Lane 2, 3, 6, 7, 8, 13, 14, 15, 18; strains with *rpo*B codon 531 susceptible alleles.

Table 2
Types of mutations in Codon and Amino acid conversion by MAS-PCR.

Gene name	codon no. in *E.coli*	Codon no. in MTB	Wild type amino acid nucleotide sequence	Mutated Codon Found	Amino acid conversion from wild type to mutated	Total Sample No.
*rpo*B	516	435	GAC	TAC	Asp>Tyr	24/213 = 11.3%
	526	445	CAC	TAC	His>Tyr	14/213 = 6.6%
	531	450	TCG	TTG	Ser>Leu	124/213 = 58.2%
	531	450	TCG	TGG	Ser>Trp	18/213 = 8.4%
			–	–	Not detected	25/213 = 11.7%

change Serine to Leucine (S450 L) while the mutation (TCG > TGG) change serine to tryptophan (S450 W). MAS-PCR failed to show amplification in 66.6% samples reflecting that the wild type sequence is absent at this position. Therefore, an additional primers were designed for codon 450 to differentiate between already reported mutations (TCG > TTG; S531 L and TCG > TGG; S531 W).

The prevalence of mutation at codon 435 was 11.3% which was lower than reported previously in Pakistan (15%) (Khan et al., 2013) and East Asia (Qian et al., 2002) (15–32%). Additionally, the prevalence of mutation at codon 445 (6.6%) was less than that reported from India (Patra et al., 2010), Argentina (Imperiale et al., 2011) and East Asia (Qian et al., 2002).

Among 33 RIF resistant isolates, various novel mutations were detected by DNA sequencing including mutations at codons 432 (Gln432Pro) and 434 (Met434Iso). Furthermore, some rare variants were detected (Met434Val, His445Asn and Leu452Pro) and a common variant was detected at codon 435 (Asp435Tyr) consistent with reports from other countries (Prammananan et al., 2008; Heep et al., 2001; Valim et al., 2000; Mani et al., 2001; Cavusoglu et al., 2002; Jou et al., 2005). However, MAS-PCR did not identify an *rpo*B 435 mutation in some replicates of certain isolates, even though this mutation was accurately identified in other isolates. A reason for this may be that the isolate contained a heterogeneous population of bacteria with mutated and wild type *rpo*B alleles, leading to amplification of the wild type PCR product.

No mutations were detected in 8 isolates by either MAS-PCR or DNA sequencing. Possible reasons for this could be mutations in a gene other than *rpo*B or mutations in an upstream regulator region of the *rpo*B gene. It is possible that mutations upstream and downstream of the 81-bp core region of the *rpo*B gene in codons 176, 553 and 541 confer resistance as has been proposed previously (Schilke et al., 1999). Alternatively, the presence of a permeability barrier could confer resistance (Schilke et al., 1999). Our limitation is that we used DNA of isolates from a culture and did not perform test directly on sputum

samples to demonstrate the additional value of the test. Only those samples were amplified that showed negativity in MAS-PCR.

5. Conclusion

We demonstrated rapid and accurate detection of rifampicin resistance mutations using MAS-PCR. This method would be feasible to implement in reference laboratories and could be used as an initial marker for screening of MDR-TB in Pakistan.

Competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.007.

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