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Molecular detection of drug resistance to ofloxacin and kanamycin in *Mycobacterium tuberculosis* by using multiplex allele-specific PCR

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ABSTRACT

Drug resistance in tuberculosis (TB) is the biggest global health challenge as it hinders the tuberculosis control program and makes the disease more worsen. Molecular methods interrupt the spread of drug resistance by facilitating the appropriate anti- tuberculosis therapy at correct time through rapid diagnosis of multi drug resistant (MDR) and extensively drug resistant tuberculosis (XDR-TB). In this study we standardized and evaluated the diagnostic utility of multiplex allele specific PCR (MAS-PCR) targeting *gyrA* D94G and *rrs* A1401G mutations for detection of resistance against two key drugs (ofloxacin and kanamycin) of second line anti tuberculosis treatment. MAS-PCR assays targeting *gyrA* D94G and *rrs* A1401G for ofloxacin (OFL) and kanamycin (KAN) resistance respectively were carried out on 150 multidrug resistant isolates of *Mycobacterium tuberculosis*. The results were compared with phenotypic drug susceptibility test against ofloxacin and kanamycin by using proportion method on MGIT 960. Of 150 MDR isolates 50 were resistant to both ofloxacin and kanamycin, 36 were resistant to ofloxacin only, 8 were resistant to kanamycin only and 56 were susceptible to both the drugs. MAS-PCR correctly identified *gyrA* D94G and *rrs* A1401G mutations in phenotypically resistant isolates with a specificity of 100%. The sensitivity of MAS-PCR was 88.66%, 93.55% and 86% for OFL, KAN and XDR-TB respectively. There was no mutation detected at *gyrA* D94G region of 12.86% (11 of 86) OFL resistant isolates while 6.89% (4 of 58) of KAN resistant isolates did not carry *rrs* A1401G substitution. MAS-PCR proves to be a rapid tool for detection of drug resistance which could also be used as an initial marker for screening of XDR-TB.

Introduction

The emergence of drug resistant *Mycobacterium tuberculosis* is the major obstacle in tuberculosis control program. WHO has reported 480 000 cases of multidrug-resistant tuberculosis (MDR-TB) estimated to have occurred in 2014 while extensively drug-resistant tuberculosis (XDR-TB) had been reported by 105 countries by the year 2015 and an estimated 9.7% of people with MDR-TB have XDR-TB [1]. Fluoroquinolones (FQs) and kanamycin are core compounds in current MDR-TB treatment regimens [2]. The delayed diagnosis and improper management of drugs has led to emergence of XDR-TB which is defined as MDR-TB with additional resistance to FQ and an injectable second-line agent kanamycin, amikacin or capreomycin [3].

Drug resistance in *M. tuberculosis* is mainly due to spontaneous mutation in the chromosomal genes of drug targets or drug modifying enzymes which is further progressed by selection of these resistant strains due to inappropriate drug therapy [4]. The common mutations associated with FQ resistance is amino acid substitution at codons 90, 91 and 94 of quinolone resistance determining region (QRDR) of *gyrA*, which encodes for α subunit of DNA gyrase [5,6]. Among the second line injectable drugs kanamycin, amikacin and capreomycin, nucleotide position 1401 and 1484 of *rrs* gene are associated with the resistance of these three drugs [7]. Studies have reported that mutation at 1402 position is also associated with capreomycin resistance while mutation at −10, −14 and −37 position of promoter region of *eis* is associated with kanamycin resistance [7,8]. Furthermore, available data suggest that D94A amino acid substitution in quinolone resistance determining region (QRDR) of *gyrA* contributes to 37% of FQ resistance and 87% of resistance in kanamycin is attributed by A1401G nucleotide substitution in *rrs* gene of 16S rRNA [6,9].

Due to slow generation time of the causative agent, patients often fail to achieve proper medication based upon the results of phenotypic drug susceptibility testing (DST). India, one of the highest TB burden countries as well as having high resistance rates to newer antibiotics, such as fluoroquinolones needs rapid and cost effective method for detection of drug resistance to impede

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Table 1
Sequences of primers used in multiplex allele-specific PCR (MAS-PCR) of *gyrA* and *rrs* gene.

Gene	Primer sequence(5′-3′)	Position	Product size	Reference
gyrA outer forward (GYRAF)	ATCGAACCGGTTGACATCGAG		391bp	In this study
gyrA outer reverse (GYRAR1)	TTTCCCTCAGCATCTCCATC			In this study
gyrA inner reverse (GYRAR2)	CCATGCGCACCAGGCTGT	D94G	256bp	Evans and Segal [14]
rrs outer forward (RRSF)	GCAACCCTTGTCTCATGTTG		438bp	In this study
rrs outer reverse (RRSR1)	GAAAGGAGGTGATCCAGCCG			In this study
rrs inner reverse (RRSR2)	GTTACCGACTTTCATGACG	A1401G	322bp	Evans and Segal [14]

the transmission of drug resistant strains [10]. Owing to facts that mutation is the most common reason of drug resistance in mycobacteria and delay in results of phenotypic DST due to long incubation time, we designed multiplex allele specific PCR targeting D94A amino acid substitution in *gyrA* and A1401G nucleotide substitution in *rrs* associated with resistance to ofloxacin and kanamycin respectively. We also evaluated the diagnostic applicability of this technique in comparison with drug susceptibility test by MGIT 960.

Material and methods

Ethics statement

This study has been ethically approved by the Institute ethical committee of Institute of Medical Sciences (ECR/526/Inst/UP/2014), Banaras Hindu University, Varanasi.

Bacterial isolates

In this study 150 non duplicate MDR strains isolated from 150 follow up patients attending indoor and outdoor facility of a tertiary care center in Varanasi were included. Such patients whose 4th month culture result was positive were considered as XDR suspects and included in this study [11]. The strains were identified as MDR by drug susceptibility testing on MGIT 960 for rifampicin, isoniazid, streptomycin and ethambutol in accordance with Revised National Tuberculosis Control Program (RNTCP) guidelines [11]. All these 150 viable isolates were further tested for resistance to ofloxacin and kanamycin at National Reference Laboratory, King George's Medical University, Lucknow, India by using the proportion method on Middlebrook 7H9 containing ofloxacin (Sigma-Aldrich) at 2 mg/L and kanamycin (Sigma-Aldrich) at 6 mg/L, in accordance with WHO guidelines [12].

Genomic DNA isolation

DNA extraction from the cultures of *M. tuberculosis* on Lowenstein–Jensen (LJ) and 1 mL of MGIT (mycobacteria growth indicator tube) culture was done by CTAB-chloroform method with some modifications [13]. The quality and quantity of DNA was analyzed by using spectrophotometer (Thermo Scientific NanoDrop 2000).

Multiplex allele specific PCR

Primers for multiplex allele specific (MAS-PCR) PCR assays were designed in house to identify *gyrA* D94G and *rrs* A1401G mutations (Table 1). PCR was performed as per the reference protocol with some modifications [14]. Briefly 20 μL reaction mixture was used containing 2.5 μL of 10X reaction buffer (GeNei, Bangalore, India), 2.5 μL of 200 μM concentrations of each of the deoxynucleoside triphosphates (dNTPs) (GeNei, Bangalore, India), 0.1 μL of 5U *Taq* DNA Polymerase (GeNei, Bangalore, India) and 1 μL of the each oligonucleotide primers GYRAF, GYRAR1 and GYRAR2 (10 pmol each) (GeNei, Bangalore, India). 5 μL (50 ng) of the DNA template and milli Q was added to maintain the final volume of 25 μL. The reaction conditions consisted of an initial denaturation step at 95 °C for 5 min followed by 5 cycles of 95 °C for 15 s, 68 °C for 5 s and 72 °C for 20 s, 5 cycles of 95 °C for 15 s, 64 °C for 5 s and 72 °C for 20 s, and 25 cycles of 94 °C for 15 s, 62 °C for 5 s and 72 °C for 20 s, followed by a final elongation step at 72 °C for 5 min. Amplification of *rrs* gene was carried out in reaction mixture of 25 μL of final volume with RRSF, RRSR1 and RRSR2 primers with same reaction condition as of *gyrA* except the annealing temperature which was 66 °C for the first 5 cycles, at 64 °C for the next 5 cycles and at 62 °C for the remaining 25 cycles. The reference strain H37Rv *M. tuberculosis* obtained from JALMA, Agra, India, was used as positive control and PCR grade water was used as a negative control.

Interpretation of agarose gel image of PCR products

Amplification of 391 and 256 bp products indicated the wild type *gyrA* while PCR products of 438 and 322 bp were obtained in case of wild type *rrs* gene (Fig. 1). The missing smaller fragments implied mutation in *gyrA* D94G and *rrs* A1401G regions. To analyze the band size, 100 bp DNA ladder (GeNei, Bangalore, India) was used.

Statistical analysis

Taking phenotypic drug susceptibility test as gold standard, sensitivity, specificity, negative predictive value, and positive predictive value of MAS-PCR with 95% confidence intervals were calculated by using an online diagnostic test evaluation tool, MedCalc.

Results

Results of phenotypic DST of 150 strains

Of 150 MDR isolates 50 were found to be XDR by phenotypic drug susceptibility test as they were resistant to both ofloxacin and kanamycin while 56 were susceptible to both the drugs. The 36 MDR isolates shown resistance to ofloxacin only and 8 were resistant to kanamycin only.

MAS-PCR was done on all these 150 MDR isolates which detected *gyrA* D94G mutation in 75(87.20%) of 86 phenotypically ofloxacin resistant MDR strains while *rrs* A1401G mutation was indicated in 52 (89.65%) of 58 kanamycin resistant MDR strains. Both *gyrA* D94G and *rrs* A1401G mutation was found in 43 of 50 XDR ($Ofx^{R}Kan^{R}$) strains (Table 2).

Comparison of DST by proportion method and MAS-PCR assay

Sensitivity and specificity values of MAS PCR in comparison with phenotypic DST by MGIT 960 for detecting resistance to OFL, KAN and XDR-TB in culture isolates are shown in Table 3. Overall

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