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DRUG DISCOVERY AND RESISTANCE

Novel mutations conferring resistance to kanamycin in *Mycobacterium tuberculosis* clinical isolates from Northern India

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SUMMARY

Twenty-nine Kanamycin resistant clinical isolates of *Mycobacterium tuberculosis* from Northern India were screened to evaluate genetic mutations in *rrs* gene, *eis* gene with its promoter, and *whiB7* gene along with its 5′UTR. 14 strains (~48.0%) collectively exhibited mutations in *rrs*, *eis* or *whiB7* target regions. While the highest frequency of mutations was found in *rrs* gene, *eis* and *whiB7* loci displayed novel mutations. The novel mutations displayed by *eis* and *whiB7* loci were found to be associated specifically with the Kanamycin resistance as none of the twenty nine Kanamycin sensitive strains harbor them. The inclusion of novel mutations of *eis* and *whiB7* loci will be useful in improving the specificity of future diagnostics.

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1. Introduction

The major obstacle to the effective control of TB is the increasing threat of resistance to the drugs used for its treatment. The emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) has raised an alarm for optimizing the existing strategies for rapid diagnosis, resistance profile determination and treatment of TB. MDR-TB displays resistance to Isoniazid and Rifampicin, with or without resistance to other first-line drugs. XDR-TB has resistance at least to Isoniazid and Rifampicin, any fluoroquinolone, and any one of the three second-line injectables i.e. Kanamycin (KAN), Amikacin (AMK) and Capreomycin (CAP). Another pattern of resistance, Pre-XDR TB, defined as resistance to Isoniazid, Rifampicin and either a second-line injectable drug or a fluoroquinolone, but not both, needs to be ruled out to forestall the progression of XDR-TB [1].

The association of mutations in genes *katG* and *inhA* with isoniazid resistance; *rpoB* with rifampin; *rpsL* and *rrs* (nucleotides 388 to 1084) with streptomycin; *embB* with ethambutol; *pncA* with pyrazinamide; *rrs* (nucleotides 1158 to 1674) and *gidB* for KAN, AMK and CAP; *eis* promoter with KAN/AMK; *tlyA* for CAP and *gyrAB* for ofloxacin have been well documented in global scenario [2–7]. In addition, WhiB7 has recently been identified as a transcriptional regulator which regulates the expression of Eis protein of *Mtuberculosis* and is reported to contribute to low-level KAN resistance indirectly [8,9].

Although some efforts have been made to study the genetics and single nucleotide polymorphisms (SNPs) related to *rrs* gene and promoter of *eis* in *M. tuberculosis* clinical isolates from Hinduja Hospital, India [2,10], studies on the second-line drug resistant clinical isolates from Uttar Pradesh, India have not been reported. Therefore, the present study was undertaken with an aim to find the overlap between resistance pattern and genetic mutations in *rrs* (Rvnr01), *eis* (Rv2416c) with its promoter, and *whiB7* (Rv3197A) along with its 5′UTR in the clinical isolates of Uttar Pradesh, India.

2. Methods

2.1. Clinical isolates of M. tuberculosis

Over a period of February 2013 to December 2014, a total of 300 laboratory confirmed *M. tuberculosis* MDR strains isolated serially were obtained from Tertiary Care Centre Tuberculosis Laboratory, Department of Microbiology, King George Medical University (KGMU), Lucknow, Uttar Pradesh, India and tested for Kanamycin

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resistance. We obtained 29 KAN resistant isolates from this repository consecutively. The cases from which these isolates were obtained were routinely referred MDR suspects from different districts of Uttar Pradesh, India. No clustering was seen as these cases were from different geographical locations, referred at different time points. Each strain corresponded to individual TB patient. The research was conducted following the national and institutional standards in accordance with the Declaration of Helsinki.

Of these 29 KAN resistant strains, 13 strains were KAN mono-resistant (44.8%) whereas 16 strains displayed cross-resistance to CAP (55.2%) and 7 strains (24.1%) exhibited cross-resistance to all the three drugs, i.e., KAN, AMK and CAP (Figure 1). The resistance profile of first and second-line drugs is mentioned in Table 1. Twenty-nine KAN sensitive strains were also selected for a similar analysis (Supplementary Table 1).

2.2. Media and determination of MIC

The clinical strains were maintained and subcultured in LJ (Löwenstein–Jensen) medium bottles regularly every 5 weeks. All the procedures dealing with *M. tuberculosis* strains were handled in the standard Biosafety Level III laboratory at KGMU.

The susceptibilities to KAN, AMK and CAP were determined according to Resazurin Microtiter Assay (REMA) [11]. Loop full of cultures corresponding to each strain, with turbidity adjusted to 1 McFarland Standard, were used to perform REMA assay as described in detail by Dixit et al. [12]. The Range of concentrations chosen for KAN/CAP and AMK were 1.25–20 μg/ml and 0.25–4 μg/ml, respectively [13,14].

Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of drug that resisted the reduction of Resazurin (change of Alamar blue to pink color indicating oxidized and reduced state, respectively), suggesting bacterial growth. The cut-off concentrations to determine resistance were $\geq$ 2.5 μg/ml for KAN/CAP and $\geq$ 0.5 μg/ml for AMK [14]. The assay was carried out in a similar approach for the KAN sensitive strains; the sole difference being the range of concentrations of the drugs used: 0.625 μg/ml to 10.0 μg/ml for KAN/CAP and 0.125 μg/ml to 2.0 μg/ml for AMK.

2.3. Isolation of genomic DNA from the clinical isolates

The genomic DNA from all resistant and sensitive strains was isolated by heat inactivation method [15]. Briefly, loop full of freshly sub-cultured colonies from each of LJ medium bottles, were resuspended in 200 μl of double distilled water. The bacteria were then lysed for 20 min by boiling at 95 °C. After cooling, 200 μl of chloroform was added and lysates were centrifuged at >13,000 g for 10 min. The lysates were then transferred to fresh micro-centrifuge tubes and used as templates for PCR after quantification by Thermo Scientific Nanodrop 1000 spectrophotometer.

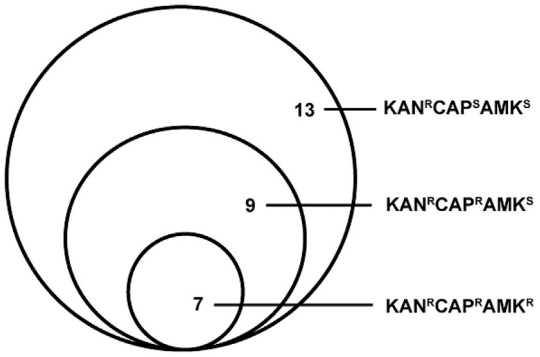

Figure 1. Cross-resistance between KAN, AMK and CAP in the 29 clinical isolates. Out of these strains, 7 and 16 isolates exhibited resistance to AMK and CAP respectively. Thirteen strains do not show cross-resistance to AMK or CAP and displayed mono-resistance to Kanamycin. All of the 7 AMK-resistant isolates were resistant to KAN and CAP. KAN, Kanamycin; AMK, Amikacin; CAP, Capreomycin; R, resistant; S, sensitive.

2.4. PCR amplification

The target fragments of *rrs* gene, *eis* gene along with its promoter, and *whiB7* gene along with its 5′UTR were amplified either by Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or *Pfu* DNA Polymerase (Promega). PCR was run using amplicon specific forward and reverse primers (0.5 μM working concentrations) designed for genes of interest (Supplementary Table 2). Based on previous reports, *rrs* gene was segregated into two regions to scan mutations in 500 bp region and 1400 bp region in the open reading frame. The PCR products were electrophoresed on 1.0% agarose gel and purified using QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced for elucidating reported and novel mutations.

2.5. DNA sequence analysis

Sequencing of PCR products was performed using Applied Biosystems analyzer with ABI BigDye v3.1 Cycle Sequencing kit. DNA sequences were analyzed by BLAST using pairwise sequence alignment (*http://www.ncbi.nlm.nih.gov/blast*). Mutations were detected in the respective genes by comparison with wild-type *M. tuberculosis* H37Rv. Validation of all mutations was done using forward as well as reverse sequencing reactions. The frequencies of occurrence of SNPs for each strain were calculated by dividing the number of genotypically resistant strains with the particular mutation by the total number of phenotypically resistant strains.

The sequenced regions of target loci containing novel as well as reported mutations were submitted to NCBI GenBank using BankIt online submission tool (*http://www.ncbi.nlm.nih.gov/genbank/submit*). The strains containing mutations in more than 1 target loci (148RN and 2009) have been named as (a) and (b). A sum of 16 sequences, corresponding to 14 strains, have been assigned the following accession numbers in February, 2015: IMT_148RNa: **KP843402**; IMT_451: **KP843403**; IMT_665: **KP843404**; IMT_702: **KP843405**; IMT_53: **KP843406**; IMT_148RNb: **KP843407**; IMT_367: **KP843408**; IMT_416: **KP843409**; IMT_915: **KP843410**; IMT_2009b: **KP843411**; IMT_2047: **KP843412**; IMT_3470: **KP843413**; IMT_3507: **KP843414**; IMT_509: **KP843417**; IMT_2009a: **KP843418**; IMT_456: **KP843419**.

3. Results

In the present study, 29 KAN resistant strains were investigated to evaluate the genetic changes that are accountable for KAN resistance in *M. tuberculosis*. Twenty-nine KAN sensitive strains were also included for comparison. For all the isolates, MICs were determined. Among KAN resistant isolates, 100% concordance for KAN (ranging from 5 μg/ml to > 20 μg/ml), 93.1% for AMK (ranging from 1 to > 4 μg/ml), and 96.6% for CAP (ranging from 5 to > 20 μg/ml) between MIC and phenotypic drug susceptibility testing (DST) was observed. 100%, 89.6%, and 93.1% coherence was observed for KAN, CAP and AMK, respectively, amongst sensitive strains ($\leq$2.5 μg/ml for KAN and CAP, and $\leq$0.5 μg/ml for AMK).

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