Deciphering the sequential events during in vivo acquisition of drug resistance in Mycobacterium tuberculosis

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ABSTRACT
Tuberculosis (TB) is caused by Mycobacterium tuberculosis (MTB) and the disease has remained a major health problem in most of the developing countries, particularly after the emergence of multidrug-resistant TB (MDR-TB). The MDR-TB is an intriguing subject and very little is known about the in vivo processes which take place during the acquisition of MDR. This study describes a unique case of pulmonary TB (PTB) from which four sequential isolates of MTB could be isolated while the patient was on anti-tubercular treatment. The first baseline isolate was sensitive to all drugs, but the subsequent three isolates acquired resistance to multiple drugs and finally the patient died after 27 months post-diagnosis when his fourth isolate became resistant to isoniazid, rifampicin, ethambutol and kanamycin. All sequential cultures were identified as MTB using conventional and molecular methods, including 16s RNA sequencing and the spoligotyping. Spoligotyping followed by comparison with SITVITWEB database revealed that all the isolates belonged to the family of the Central Asian Strain Delhi (CAS1_Delhi, ST26) genotype, and no cross or mixed infections were observed. The drug resistance was further characterized at the molecular level by sequencing the target genes (katG, inhA, rpoB, embB, eis promoter region and rrs). The results revealed mutated alleles associated with resistance to the respective drugs. This unique case indicates that it is possible to isolate MTB during treatment if the strain is acquiring resistance. The data presented from four sequential isolates provides an insight into what sequential genetic and proteomic changes occur in the bacteria during the in vivo acquisition of MDR.

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Introduction
Mycobacterium tuberculosis (MTB) is the most successful human pathogen worldwide causing an estimated 8.7 million new cases and more than 1.4 million deaths annually [1]. India has the highest estimated burden of tuberculosis (TB) in the world, accounting for 26% of all TB cases worldwide. The emergence of multidrug-resistant (MDR) strains of MTB...
A 22-year-old male patient was diagnosed with pulmonary TB from a sensitive strain of MTB. The patient from Delhi, India, and summarizes the data on their previous anti-TB treatment [6]. It is also reported that an increasing frequency of drug resistance observed in the CAS1_Delhi isolates was not linked to the patients’ history of previous anti-TB treatment [6].

This study describes a unique case of four sequential isolations of MTB CAS1_Delhi genotype from a 22-year-old male patient from Delhi, India, and summarizes the data on their genotypes, drug resistance and possible evolution of MDR-TB from a sensitive strain of MTB.

Materials and methods

The patient and MTB isolates

A 22-year-old male patient was diagnosed with pulmonary TB on the basis of clinical and radiological findings, and sputum samples were referred from designated microscopy and DOTS center to the Tuberculosis Laboratory of the Clinical Microbiology and Molecular Medicine Division, All India Institute of Medical Sciences, New Delhi, India, for culture and drug susceptibility testing. The patient was prescribed with category I anti-tubercular treatment (ATT) under directly observed treatment-short course (DOTS) program. The treatment comprised of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), also known as category I treatment as per revised national TB control program (RNTCP), an initiative of the Government of India. During the intensive phase of DOTS, the four drugs are administered for two months, followed by four months of treatment with only two (INH and RIF) drugs.

However, this patient did not follow the regimen, and he had several interruptions in the treatment, particularly after the intensive phase when his general condition improved and he became asymptomatic. Several sputum samples were collected during the follow-up period of treatment and subjected to BACTEC-MGIT-960 culture isolation. The base line culture was positive, and the isolate was identified as MTB by conventional phenotypic characteristics and confirmed by an in-house polymerase chain reaction (PCR) method [7]. This culture was labeled as isolate A. After three months of cessation of treatment (6 + 3 = 9 month, Fig. 1), his condition again deteriorated and his sputum sample was again positive for MTB. This second culture was labeled as isolate B. He was again prescribed with INH, RIF, PZA, EMB and streptomycin (STR). This regimen is known as category II as per the RNTCP, India guidelines. Within two months, his clinical condition again improved, but after a gap of four months, his symptoms reappeared. His sputum was again culture positive which was labeled as isolate C. The patient was once again prescribed with the same treatment for another 12 months, but this time also he stopped treatment after six months. After that, his condition further deteriorated and he died of disseminated disease. By this time 27 months had elapsed (Fig. 1). Before his death, a fourth isolation was made from his sputum sample and labeled as isolate D.

All the four clinical isolates (A, B, C and D) were identified as MTB using standard protocols followed in our laboratory, which is accredited for culture, drug susceptibility and line probe assay [7,8] and also by 16s RNA gene sequencing. The anti-mycobacterial drug susceptibility testing was performed on all the isolates by BACTEC-MGIT-960 (Becton Dickinson, Microbiology Systems, Sparks, MD), singly as well as in pairs. To determine the minimum inhibitory concentration (MIC), tetrazolium microplate assay (TEMA) and proportional method using Middlebrook 7H10 (Difco, Detroit, MI, USA) agar plates were used against first-line drugs. In proportional method the medium contained STR (2.0 μg/ml), INH (0.2 μg/ml), RIF (1.0 μg/ml), and EMB (6.0 μg/ml) [7,9,10].

From all cultures, DNA was extracted and subjected to spoligotyping using a commercial kit (Ocimum Biosolutions, India) as per the manufacturer’s instructions [11], and isolates were identified using the international SITVITWEB database [12,13]. Further, 24-loci MIRU-VNTR was performed by PCR amplification of individual loci using specific primers as described previously [14]. The sequencing of 16sRNA for the rpoB, inhA, katG, embB, eis and rrs gene targets was done using the primers as described elsewhere [6,15–18].

Results and discussion

Isolates, resistance pattern and gene mutations

A 22-year-old male patient from Delhi was suspected of suffering from pulmonary TB (PTB) based on clinical history (cough, fever, chest pain, weight loss and loss of appetite), tuberculin skin test (16 mm) and chest X-ray done in AllIMS hospital. The chest X-ray showed bilateral cavitory lesions. The patient had a family history of contact with his brother who was a smear-positive case and who died of TB a few years ago. However, no isolation from his brother’s samples was attempted, as it was not required to culture the samples under the national TB control program at that time. The initial result of Ziehl-Neelsen (ZN) staining of sputum was positive for acid-fast bacilli (AFB). Culture isolation and the drug susceptibility tests were performed using BACTEC-MGIT 960 (BD), as per the World Health Organization (WHO) guidelines. The culture was identified as MTB by conventional phenotypic characteristics and confirmed by an in-house PCR method [8]. The patient was administered free anti-tubercular treatment (ATT) under the DOTS program of the Government of India. (Please see methods). Three more culture isolations were made during the course of the treatment period, albeit at irregular intervals, and these sequential isolates were la-
beled as isolates A, B, C & D, respectively, as mentioned in the material and methods section (Fig. 1).

The initial isolate (A) was sensitive to all of the four first-line drugs (STR, INH, RIF and EMB), but the consecutive isolates (isolates B, C and D) were found to be resistant to these drugs. The MIC of isolate B increased significantly to INH, RIF and EMB, however it was still sensitive to kanamycin. Significantly, the third and fourth isolates were found to be resistant not only to INH, RIF and EMB, but also to kanamycin (Fig. 1). Infection with multiple strains or re-infection with other resistant strains during the course of treatment is common in endemic countries like India; hence, to confirm the clonal nature of all the four isolates, spoligotyping was done, and the results confirmed that all the isolates belonged to the family of Central Asian Strain Delhi (CAS1_Delhi, ST26) genotype in the SITVITWEB database. The 24-loci MIRU-VNTR patterns for all four isolates were identical and confirmed that all isolates belonged to the same family with identical MIRU-VNTR profiles (Fig. 1). The sequencing of the rpoB (RIF), katG, inhA (INH), rrs and eis promoter regions (Kan') revealed mutated alleles associated with resistance to the INH, RIF, and EMB, but no mutation was found for kanamycin resistance, as shown in Fig. 1. For INH resistance, katG 315 G-C, katG 463 T-G, InhA-15 C-T and rpoB 531 (C-T), rpoB 531 (C-T), embB 294 (G-A), eis promoter region and rrs gene have no mutation.

The worldwide distribution of ST26 strains was essentially confined to the Middle East and Central Asia, more specifically in the Indian subcontinent (75%) [3,4]. Hence as expected, the isolates also belonged to this most prevalent genotype.

There are only a few studies where differential MIC pattern and genetic mutations have been reported in drug-sensitive and drug-resistant isolates. Even these studies have been carried out on isolates obtained from different patients [19,20]. Such isolates, therefore, do not provide deeper insight into
the phenotypic and genotypic sequential events that occur in MTB under in vivo conditions. Only recently, Saunders et al. [21] reported a similar case in which they could isolate sequential isolates from a patient on anti-tubercular treatment, but their strategy of comparing sensitive and resistant isolates was different from the one used in this study. They only did deep sequencing of the isolates and found known mutations in katG and rpoB genes. Another study was published in 2012 from China [22]. However, the most interesting finding in this case is resistance selection for 4 drugs within 27 months. This is alarmingly the fastest selection process to best of this research’s knowledge. The speed at which the strain turned from pan-sensitive to multidrug resistant is highly disturbing for TB control programs and indicates that any mixed population at any stage, such instances are common but not frequently feasible to study. It is obvious from the case history of our patient that the mycobacteria remained under a constant intermittent drug pressure, which is considered to be the most suitable condition for selection of resistant strains in vivo [22]. However, the most interesting finding in this case is resistance selection for 4 drugs within 27 months. This is alarmingly the fastest selection process to best of this research’s knowledge. The speed at which the strain turned from pan-sensitive to multidrug resistant is highly disturbing for TB control programs and indicates that more personalized counselling and awareness of strict adherence to the regular drug regimens cannot be compromised. This case also emphasizes the urgent need for compulsory isolations and forced treatment in such non-cooperative patients to contain the fast spread of MDR-TB.

Author contribution

AS & KG carried out the experiments; NS provided patient patients to contain the fast spread of MDR-TB.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The study was funded by the Indian Council of Medical Research, New Delhi, but the funding agency had no role in this subject and study design, data collection, analysis, decision to publish, or preparation of the manuscript.

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