Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/IJMYCO

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

Amit Singh [a], *Krishnamoorthy Gopinath* [a,1], *Niti Singh* [b,2], *Sarman Singh* [a,*]

[a] *Division of Clinical Microbiology and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India*

[b] *DSDC Designated Microscopy and DOTS Center, Shahpur Jat, New Delhi, India*

ARTICLE INFO

Article history:
Received 1 October 2013
Received in revised form
16 October 2013
Accepted 17 October 2013
Available online 15 November 2013

Keywords:
Mycobacterium tuberculosis
MDR-TB
CAS1_Delhi genotype
Spoligotyping
Sequential isolates

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.

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

* Corresponding author. Address: Division of Clinical Microbiology and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029, India. Tel.: +91 11 26588484; fax: +91 11 26588641.
E-mail address: sarman_singh@yahoo.com (S. Singh).
[1] Present address: Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa.
[2] Present address: Department of Microbiology, National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India.
2212-5531/$ - see front matter
http://dx.doi.org/10.1016/j.ijmyco.2013.10.006

is threatening to make TB one of mankind's most pervasive infectious diseases incurable. These drug-resistant strains are more infectious by virtue of their high transmissibility in the population [2]. There are several genotypes of MTB.

MTB CAS1_Delhi lineage was first identified in Delhi region but now it is an emerging pathogen in several areas of the world, predominantly in South East Asian Countries. It belongs to the Spoligotype International Types (SIT) 26, 25, 141, 381, 1327, 1343 and 1344 CAS1_Delhi type (ST 26), and is highly prevalent in the Indian subcontinent [3,4]. In Europe and Australia, these strains were often found to be associated with migrants from South Asia [5]. The lineage, however, is not considered to be more prone to resistance, as is the Beijing strain. When resistant, mutations in the *rpoB*, *katG* genes are more frequent in these isolates as compared with non-CAS_Delhi isolates [6]. It is also reported that an increasing frequency of drug resistance observed in the CAS1_Delhi sub-lineage 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 AIIMS hospital. The chest X-ray showed bilateral cavitary 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-