Original Article

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Microbiology Section

Effect of *Mycobacterium tuberculosis* Lineage, Drug Resistance and HIV Status on the Outcome of Patients with Tuberculous Meningitis

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ABSTRACT

Introduction: Tuberculous Meningitis (TBM) is a devastating disease with high morbidity and mortality. Resistance to anti tubercular drugs, strain variation among *M. tuberculosis* and HIV status of the host are important factors governing the disease progression in pulmonary tuberculosis and data regarding TBM is lacking. The geographical variations present in these factors necessitate local epidemiological studies.

Aim: The present study was conducted to assess the influence of drug resistance and lineage on the outcome of HIV positive and negative cases of TBM.

Materials and Methods: Genotypic profiling using 24-loci Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) analysis was retrospectively conducted on 70 (8 HIV positive patients, 62 HIV negative patients) Cerebrospinal Fluid (CSF) culture isolates of *M. tuberculosis* processed in the Mycobacteriology laboratory of PGIMER during January 2010-December 2015. Drug susceptibility was performed phenotypically by 1% proportion method and genotypically by rpoB and katG gene sequencing.

Results: Total 4 (5.7%) out of 70 isolates of *M. tuberculosis* were multidrug resistant and were associated with higher mortality than the drug sensitive ones. Among the different lineages, the Beijing genotype was uniformly associated with drug resistance and mortality. All HIV positive patients had a poor outcome, irrespective of drug resistance and lineage.

Conclusion: Multidrug resistance lineage of *M. tuberculosis* and HIV status are important determinants of mortality in patients of TBM. Targeting these factors can contribute to a favourable outcome.

Keywords: Beijing genotype, Multidrug resistant tuberculosis, Tuberculosis-HIV coinfection

INTRODUCTION

Tuberculous meningitis is the most severe form of Extra Pulmonary Tuberculosis (EPTB) in highly endemic, low resource countries like India [1]. Coendemicity of tuberculosis and HIV/AIDS in these countries contribute to poor outcome in patients with TBM. Despite the availability of highly effective Anti Tubercular Therapy (ATT), the outcome is poor with high mortality rates (30% in HIV negative to 60% in HIV positive cases) and neurological sequelae [2,3]. A recent study has shown that the Central Asian family (CAS)-type is the most predominant lineage of *M. tuberculosis* in North India followed by East African Indian (EAI), Beijing, Haarlem and T-type [4]. Important factors that contribute to unfavourable outcomes in TBM cases are genetic variability of *M. tuberculosis* and emergence of drug resistance [1,4]. While drug resistance is increasing at an alarming rate in patients with TBM, evidence is accumulating that certain lineages like the Beijing strain of *M. tuberculosis* may have a graver prognosis [4-7]. These factors are well studied in PTB, however there is a paucity of data in patients with TBM [8]. Knowledge of mycobacterial lineage and drug resistance in TBM patients will not only help in establishing the molecular epidemiology of circulating strains but will also help in developing effective control strategies. Both these factors show wide geographical variation and hence, studies evaluating local epidemiological data are necessary [9]. The present study was, therefore, designed to investigate the contribution of the circulating lineages, drug resistance patterns and HIV status on the outcome of patients of TBM in our region.

MATERIALS AND METHODS

Study design: A retrospective study was carried out on 70 previously collected culture isolates of CSF samples from patients with TBM, processed in the Mycobacteriology laboratory of PGIMER, Chandigarh, India during January 2010-December 2015. Out of these 70 isolates, eight were isolated from CSF samples of HIV positive patients (HIV status was determined by ELISA as per NACO recommendations and the remaining 62 from HIV negative patients [10]. The male:female ratio was 8:0 for HIV positive patients and 41:21 for HIV negative patients. Samples for which pure growth on culture media was obtained were included in the study. Samples growing contaminated cultures or those who were already started on ATT were excluded. The study was approved by our Institutions Ethics Committee. The samples were obtained before initiation of ATT. Samples having pure growth on culture tubes were included while those showing contamination were excluded.

Processing of CSF samples: In the present study, 1 to 2 mL of CSF was collected from each patient and processed according to the standard bacteriological procedures for *M. tuberculosis* 200 µL of centrifuged pellet was separated for DNA extraction and another 200-300 µL was stored at -20°C for future use [11]. A part of the sample was inoculated onto two slants of in-house prepared Lowenstein Jensen (LJ) medium incubated at 37°C for eight weeks and two BACTEC MGIT 960 bottles each, the latter being processed as per the manufacturer's instructions. (Briefly, 0.5 mL of concentrated sample and 0.8 mL of MGIT growth supplement were added aseptically to MGIT tubes which were then incubated aerobically at 37ºC for maximum six weeks).

DNA extraction and identification of *M. tuberculosis*: DNA extraction from both LJ medium and BACTEC MGIT 960 was carried out by Cetyl Trimethylammonium Bromide (CTAB)-phenol-chloroform method, as described previously [12]. M. tuberculosis

was confirmed using a multiplex PCR method that was standardised at our institute [13].

Drug Susceptibility Testing (DST): DST was done using both genotypic and phenotypic methods. Genotypic DST was carried out for rifampicin and isoniazid. A 256 bp region of rpoB gene containing the 81 bp core sequence of the rpoB gene and a 166 bp region of the katG gene containing codon 315 were amplified using gene specific primers pairs [14]. Molecular grade water was used in the PCR negative control reactions in each run. Phenotypic DST was done using the 1% proportion method on LJ medium and the BACTEC MGIT 960 method (BD Microbiology systems). H37Rv strain of M. tuberculosis was used as the positive control strain. Four drugs-rifampicin, isoniazid, ethambutol and streptomycin were tested, as described previously [15].

Sequencing for rifampicin and isoniazid: To look for various types of mutations, sequencing was carried out for rpoB gene and katG gene for rifampicin and isoniazid resistance, respectively, as reported earlier by our center [14,15]. Amplicon sequencing was carried out as per the manufacturer's instructions using the Big-Dye v3.1 Terminator Cycle sequencing kit (Applied Biosystems, USA). The products were analysed using an ABI 3130 Genetic Analyser (Applied Biosystems, USA).

MIRU-VNTR genotyping of *M. tuberculosis*: Extracted DNA was subjected to amplification at 24 genetic loci using standard primers [16]. The genotypes were determined using a reference database at www.miru-vntrplus.org, as described previously [17]. All 70 CSF culture isolates were evaluated for drug susceptibility profile and MIRU-VNTR genotyping. Patient HIV status was concealed so as to blind the investigators.

RESULTS

CSF Culture Isolates from HIV Positive Patients with TBM

DST profile: In the HIV positive culture isolates, 2 (25%) out of 8 isolates of M. tuberculosis were Multi Drug Resistant (MDR) while, five out of eight (62.5%) were pansensitive. DST revealed mono-rifampicin resistance in one isolate. Sequencing of the rpoB gene demonstrated mutation at codon 531 in two MDR isolates and at codon 533 in the mono-rifampicin resistant isolate. Isoniazid resistance was contributed by a well known mutation at codon 315 of the katG gene.

Genotyping: Both the MDR strains were of the Beijing type whereas one strain which was rifampicin mono-resistant was a CAS-Delhi type. Out of five pansensitive CSF culture isolates, three out of five (60%) were attributed to the CAS-Delhi type and two out of five (40%) to the EAI type.

Outcome: Among the eight CSF isolates from HIV positive patients with TBM, 4 (50%) out of 8 experienced a poor outcome i.e., they succumbed to death. Out of these four cases, two were the Beijing strains and two were the CAS-Delhi strains. 2 (25%) out of 8 cases of the EAI genotype responded well to treatment and had favourable outcome i.e., they were alive and healthy. Remaining 2/8 (25%) were lost to follow-up, both being the CAS-Delhi type [Table/Fig-1].

CSF Culture Isolates from HIV Negative Patients with TBM

DST profile: In the present study, 54 (87.1%) out of 62 isolates from HIV negative group were susceptible to all the drugs tested while drug resistance was present in 8 (12.9%) out of 62. Out of these eight drug resistant isolates, 2 (3.2%) out of 62 were MDR being resistant to both rifampicin and isoniazid. 3 (4.8%) out of 62 isolates were mono-resistant to isoniazid and one each (1.6%) was mono-resistant to rifampicin and ethambutol. There was one isolate that was resistant to both isoniazid and ethambutol. Rifampicin-resistant isolates demonstrated mutation at codons 531, 533 and 516 of rpoB gene and isoniazid-resistant isolates at codon 315.

Genotyping: Out of the two MDR isolates, one belonged to CAS-Delhi type and other was Beijing type. Out of three isoniazid mono-resistant isolates, two were CAS-Delhi type and one was EAI type. Ethambutol mono-resistant isolate was Haarlem type. Among the 54 pansensitive isolates, 27 (50%) out of 54 were CAS-Delhi type, 13 (24%) out of 54 were EAI type, 6 (11.1%) out of 54 were T-type, 5 (9.2%) out of 54 were Haarlem type and 3 (5.5%) out of 54 were untypeable.

Outcome: Among the eight cases of drug resistant TBM, seven had poor outcome. Out of these, two were MDR (one Beijing type, one CAS-Delhi type), three were isoniazid mono-resistant (two CAS-Delhi type, one EAI type) and one was rifampicin mono-resistant (CAS-Delhi type). The one isolate with both isoniazid and ethambutol resistance (CAS-Delhi type) also had a poor outcome. The single ethambutol mono-resistant isolate is still doing well. Among the 54 cases of drug sensitive TBM, 37 (59.9%) patients had a good outcome. 21 (33.8%) out of 54 HIV negative patients

Genotype	Total no.	Drug susceptibility profile			rpoB Gene		katG	Outcome		
		MDR	mono-Rif	PS	c 531	c 533	c 315	LFU	A	D
CAS-Delhi	4	-	1	3	-	1	-	2	-	2
EAI	2	-	-	2	-	-	-	-	2	-
Beijing	2	2	-	-	2	-	2	-	-	2
Total, n (%)	8	2 (25%)	1 (12.5%)	5 (62.5%)	2	1	2	2 (25%)	2 (25%)	4 (50%)

[Table/Fig-1]: Lineage, drug susceptibility profile and outcome of eight HIV-positive cases of TBM.
MDR: Multidrug resistant; Rif: Rifampicin; PS: Pansensitive; c: Codon; LFU: Left to follow up; A: Alive; D: Death; TBM: Tuberculous meningitis

Genotype	Total no.	Drug susceptibility profile						rpoB gene			katG	Outcome		
		MDR	mono-Rif	mono-Inh	mono-Etm	Inh+Etm	PS	c 516	c 531	c 533	c 315	LFU	A	D
CAS-Delhi	32	1	1	2	-	1	27	1	-	1	3	4	14	14
EAI	14	-	-	1	-	-	13	-	-	-	1	-	10	3
T-type	6	-	-	-	-	-	6	-	-	-	-	-	6	-
Haarlem	6	-	-	-	1	-	5	-	-	-	-	-	6	-
Beijing	1	1	-	-	-	-	-	-	1	-	1	-	1	1
Untypeable	3	-	-	-	-	-	3	-	-	-	-	-	-	3
Total, n (%)	62	2 (3.2%)	1 (1.6%)	3 (4.8%)	1 (1.61%)	1 (1.6%)	54 (87.1%)	1	1	1	5	4 (6.4%)	37 (59.9%)	21 (33.8%)

[Table/Fig-2]: Lineage, drug susceptibility profile and outcome of 62 HIV negative cases of TBM.
MDR: Multidrug resistant; Rif: Rifampicin; Inh: Isoniazid; Etm: Ethambutol; PS: Pansensitive; c: Codon; LFU: Left to follow up; A: Alive; D: Death; TBM: Tuberculous meningitis

with TBM had a poor outcome and 4 (6.4%) out of 54 were lost to follow-up, as shown in [Table/Fig-2].

Effect of Drug Resistance and Genotype on Clinical Outcome of Patients with Known HIV-Status.

DST profile: In the current study, 11 (15.7%) out of 70 isolates were resistant to at least one of the tested drugs. A total of 4 (5.7%) out of 70 were MDR, being 2 (25%) out of 8 in HIV positive and 2 (3.2%) out of 62 in HIV negative patients. Mortality was significantly higher in drug resistant cases (more than 87%) as compared to drug sensitive ones (less than 30%), irrespective of HIV-status and genotype.

Genotyping: Beijing genotype was consistently associated with drug resistant profile and mortality, irrespective of the HIV status of the patient. CAS-Delhi lineage contributed to an overall mortality in 16/36 (44.4%) patients. Mortality in EAI lineage was observed only in HIV negative patients, irrespective of drug susceptibility profile. The three untypeable isolates were fatal even in immunocompetent host despite being pansensitive. Haarlem genotype was associated only with HIV negative patients and was neither drug resistant nor fatal, barring one isolate which had ethambutol mono resistance and a favourable outcome.

Outcome: Overall, 25 (35.7%) out of 70 of the study patients died, 39 (55.7%) out of 70 had a favourable outcome and 6 (8.5%) out of 70 were lost to follow-up. Among the patients that could be followed up, mortality was 100% among the four HIV positive cases of TBM, irrespective of the genotype-and resistance profile [Table/Fig-3].

Drug susceptibility pattern	Drug resistant		Drug sensitive		Total deaths	Lineage-based mortality (n/N)
	HIV+	HIV-	HIV+	HIV-		
CAS-Delhi	1	5	1	9	16	44.4% (16/36)
EAI	-	1	-	2	3	18.7% (3/16)
T-type	-	-	-	-	-	-
Haarlem	-	-	-	-	-	-
Beijing	2	1	-	-	3	100% (3/3)
Untypeable	-	-	-	3	3	100% (3/3)
Total deaths	3	7	1	14	25	
Drug susceptibility profile-based mortality (n/N)	100% (3/3)	87.5% (7/8)	20% (1/5)	25.9% (14/54)	35.7% (25/70)	

[Table/Fig-3]: Contribution of drug susceptibility pattern and lineage on the unfavourable outcome in patients of TBM.
n: Number of deaths; N: Number of cases; TBM: Tuberculous meningitis

DISCUSSION

Mycobacterial genotyping tools like MIRU-VNTR can be used to analyse the incidence, distribution and transmission patterns of different strains of *M. tuberculosis*. With this background knowledge of circulating strains, region specific infection control strategies and vaccine development attempts can be undertaken pertaining to a particular geographical area. Although several studies have addressed the issue of prevalence of different lineages of *M. tuberculosis* in different parts of India, data regarding their association with patient outcome is largely lacking [17-23]. Moreover, since most of the studies have been conducted for PTB, there is a dearth of data for EPTB especially TBM [17,19,21,22]. Therefore, in this study, we first analysed TBM cases (both HIV positive and HIV negative) for their genotype and drug resistance pattern, and then evaluated the effect of these variables on the outcome of the patients.

The most prevalent genotype among our TBM cases was CAS-Delhi 36 (51.4%) out of 70, followed by EAI 16 (22.8%) out of 70. T-type and Haarlem contributed 6 (8.5%) out of 70 cases each while Beijing and untypeable genotypes contributed 3 (4.2%) out of 70 case each. This is in accordance with other studies from North India wherein CAS-Delhi was found to be the most common genotype ranging from 46% in PTB to 57% in EPTB [17,18]. Interestingly, this particular genotype is widely present in North India only. While it accounted for 41% isolates in North-East India (Dibrugarh), 39% isolates in Western India (Mumbai), mere 11% isolates in Central India (Varanasi), its incidence was found to be around 3% in South India [19-22]. CAS-Delhi genotype was also associated with an appreciable mortality rate of 44.4%.

The EAI genotype, the second most frequently isolated lineage in the present study shows a more drastic geographical difference between South India and the rest of India. It solely contributes to as high as 84% isolates of *M. tuberculosis* in South India while at all other regions the prevalence is more uniform varying between 21 to 33% [17,18,20,23]. The 22.8% prevalence in the current study also lies within the same range. This North-South discrepancy in CAS and EAI lineages is a well known phenomenon among Indian isolates of *M. tuberculosis* and is believed to be due to the remarkably different origin and evolution of the inhabitants of these two geographical areas. Knowing the genotype of the infecting strain, thus, can help in keeping a check on the transmission of *M. tuberculosis*.

In the present study, 4.2% of the isolates harbored Beijing genotype. The reported incidence from other parts of the country varies from 35% in 5-10% in North India, North-East, 19% in Western and 21% in Central [17,19-21]. Beijing genotype has global distribution with its MDR strains spreading throughout central Asia and Europe [24]. It was the most common genotype isolated from TBM cases in Thailand (56%) and EPTB cases in USA (89%) [25,26]. This genotype has been notorious for its high transmission capacity, better host adaptability, higher virulence and propensity for MDR [4]. In the present study also, all Beijing types were MDR and all patients lost their lives irrespective of HIV status. Earlier reports from India revealed that Beijing genotype was MDR in 13 (62%) out of 21 isolates in PTB and 7 (64%) out of 11 in EPTB [18,21]. The fact that this genotype was associated with mortality in 100% of the patients, highlight the importance of early recognition and genotype specific management of such cases.

The Haarlem genotype was found only in 8.5% isolates among our TBM patients. Although, specific data in this regard is lacking from other regions of the country, a recent review also mentions 3% prevalence of Haarlem strains in India; Hungary and China being the countries with highest and lowest prevalence, respectively [27]. T-type genotype was also detected in 8.5% of the total isolates, all of which had a good outcome. This incidence is similar to another study from North India that has reported genotype-T in 7/60 (11.6%) cases of TBM [18]. The point of difference is, unlike in our study where all T-type were pansensitive, two out of seven were MDR in that study.

Among the 70 study isolates, 11 (15.7%) exhibited drug resistance to first line ATT drugs like rifampicin, isoniazid and ethambutol. The rate of MDR was 5.7% (4/70) in the present TBM study. This is in accordance with earlier studies from Asia reporting MDR in TBM in the range of 5% in Thailand to as high as 32% in China [25,28]. The presence of Multidrug Resistant Tuberculosis (MDR-TB) was independent of the HIV status of patients in the present study. Conflicting results exist in the world literature in this regard. On one hand, the treatment outcomes were found to be worse in HIV positive patients of TB than HIV negative ones (2014), possibly due to presence of MDR; while on the other hand, another study demonstrated that HIV positive patients were 78% less likely to have MDR-TB [29,30]. The following could be the possible explanations: firstly, MDR infection is more of an epidemiological and therapeutic misadventure than an outcome of host immune response. While the endemicity of circulating MDR strains in a geographical location determine the cases of primary drug resistance, the mutational

changes occurring consequent to non compliance to therapy lead to secondary drug resistance. The host immune status, thus, doesn't have much role to play. Secondly, both the studies with contradictory results have been carried out on patients with PTB. Possibility exists that an increased association was found due to increased risk of droplet transmission of MDR strain from the immunocompromised host than by the increased susceptibility per se. Thirdly, reactivation of an otherwise latent MDR strain in the background of either severe immunosuppression or immune reconstitution could have led to such observations.

Although, development of MDR-TB infection was not associated with HIV status in this study, the presence of MDR-TB was uniformly fatal in HIV positive population. This is in accordance with previous studies that have also documented poor clinical outcomes when HIV positive patients of TBM are started on standard ATT [31]. Strikingly, the overall mortality did not show drastic difference due to the HIV status of the patients, being 50% (4/8) in HIV positive group and 33.8% (21/62) in HIV negative group. This is in accordance with a recent randomised controlled trial reporting that HIV positive TBM patients had no statistically significant difference in the outcome than HIV negative TBM patients [2].

Mono resistance to isoniazid was constantly associated with mortality. Its association even in HIV negative patients, irrespective of lineage, provides evidence that it was an independent predictor of mortality. Similar finding was noted in a study by Tho DQ et al., wherein the adjusted hazard ratio was 1.78 for patients with isolated isoniazid resistance [31]. The mortality resulting from this association develops at around three months of ATT when the two drugs of intensive phase, pyrazinamide and ethambutol, are stopped and the patient is practically only on rifampicin. Rifampicin resistant subpopulations seize this opportunity to gain ground leading to therapeutic failure and clinical deterioration. To combat such a situation, addition of an extra mycobactericidal drug would be more prudent than extending the duration of intensive phase.

This is the first study of its kind which has evaluated two important predictors of clinical outcome in TBM: drug resistant profile and bacterial lineage, on both HIV positive and HIV negative patient populations. The study also unfolds several research questions: 1) whether the lineage of *M. tuberculosis* has any bearing on reinfection or relapse of the disease; 2) utilising whole genome sequencing approach to decipher intra-lineage variations up to SNP level; 3) determine what host factors are involved or what immune responses are evoked by different lineages/Single Nucleotide Polymorphisms (SNP) types, and finally, develop rapid methods for diagnosing impending resistance and customising ATT depending upon the host immune status and offending SNP of the organism.

LIMITATION

The limitation of the study is the sample size which is small enough to have comparable values for all observations and to apply any statistical tests. This is, however, justified by the fact that it is indeed rare to have an HIV positive patients presenting with TBM, that too with *M. tuberculosis* being isolated in culture. Also, considering the high morbidity and mortality associated with TBM, a study involving other factors that contribute towards the overall outcome of the patient is indispensable. This study highlights that drug resistance (multidrug or mono-rifampicin or mono-isoniazid), lineage of *M. tuberculosis* (Beijing>CAS>EAI) and HIV status of the host are important determinants of mortality in patients with TBM. An understanding of their contribution in the disease pathogenesis can lead to a favourable outcome in an otherwise gloomy prognosis of TBM.

CONCLUSION

The study concludes that certain predictors are associated with higher mortality in patients of TBM. The important ones among them are MDR strain and HIV-coinfection. Among the different genotypes of *M. tuberculosis*, the Beijing genotype is associated with highest mortality followed by CAS-Delhi.

Author contribution: Concept of study: Kusum Sharma, Manish Modi; Sample collection: Kusum Sharma, Sandeep Sharma, Pallab Ray, Manoj Goyal, Niranjan Khandelwal; Microbiological investigations: Kusum Sharma, Sandeep Sharma; Analysis: Kusum Sharma, Megha Sharma, Aman Sharma; Drafting of manuscript: Kusum Sharma, Megha Sharma, Aman Sharma, Paul D. Fiorella; Study supervision: Subhash Varma.

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