

Analysis of mutational pattern in multidrug resistant tuberculosis (MDR TB) in a geographically isolated northeastern region of India

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Abstract: Objectives: To assess the mutational pattern of multidrug resistant tuberculosis and evaluate the performance of MTBDRplus and MTBDRsl in rapid detection of multidrug and second line resistance of tuberculosis in a geographically isolated population.

Methods: The assay was performed directly on 375 smear positive sputum specimens from patients referred to a busy routine diagnostic lab in Manipur, northeast India. MTBDRplus and MTBDRsl were performed as per the directions of the manufacturer. Results were compared with BacT/Alert 3D culture and DST system

Results: Of the 375 smear positive specimen, 364 (97.1%) gave interpretable results by MTBDRplus assay within 1 – 2 days with an additional 2days for EMB resistance of the MDR strains. Sensitivity, specificity, and positive and negative predictive values were high for rifampicin and multidrug resistant strains with a range of 98 – 100 %. Isoniazid has comparatively lower sensitivity of 81% amongst the drug compared with phenotypic BacT/Alert 3D results. The mutational pattern of this geographically isolated region didn't have much variation as shown by the absence of bands in the *rpoB* MUT2A/2B and *inhA* MUT2/3A/3B regions of the gene.

Conclusions: The results of this study supports the use of MTBDRplus for rapid diagnosis of TB from smear positive specimens considering the drastic reduction of time in diagnosis and its accuracy being comparable to phenotypic methods while the MTBDRsl's ability to detect rare mutations makes it a potential method for analysing mutational patterns.

Keywords: tuberculosis; MTBDRplus; MTBDRsl; northeast India; Manipur

I. Introduction:

India has become the world hub for tuberculosis. According to the latest World Health Organisation (WHO) report titled "Global Tuberculosis Control 2011" 8.8 million cases of TB are registered of which 2.5 million were from India. India alone accounted for an estimated 26 % of all TB cases worldwide and remains a major killer, killing 2 persons every 3 minutes which is nearly 1000/day.

Multidrug resistant tuberculosis (MDR TB) has evolved to become a serious threat to the general public due to limited treatment options (I-Ching et al, 2006; Morgan et al, 2005). In order to have efficient treatment, it becomes imperative that detection be done as early as possible. The emergence of extensively drug resistant tuberculosis (XDR TB) looms large in the backdrop of such scenario with further spread of drug resistance, especially in HIV-infected patients, as was recently reported (Gandhi 2006). XDR TB, defined as MDR TB with additional resistance to fluoroquinolone antibiotic and atleast one of the three injectable drug used for MDR TB treatment (Centre 1 & 2) has been identified in 84 countries with an average proportion of MDR TB cases with XDR TB as 9% (6.7 – 11.2%) (WHO, 2012).

As such early detection of drug resistances constitutes one of the priorities of TB control programs. Detection of drug resistance has been performed in the past by conventional methods based on detection of growth of *M.tuberculosis* in the presence of antibiotics. Conventional indirect methods of detection for drug resistance take 10 – 12 weeks for the sample to get drug susceptibility test (DST) report while direct method may be given by 4 – 6 week (Canetti et al, 1963; Caviedes et al, 2002; Kent & Kubica, 1985). However due to the labouriousness of some of these methods and most of all the long period of time necessary to obtain results, new technologies and approaches have been proposed which include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of rifampicin (RIF) resistance, since it is considered a good surrogate marker for MDR TB, especially in settings with high prevalence of MDR TB (I-Ching et al, 2006; Morgan et al, 2005). Genotypic methods have the advantage of a shorter turnaround time, no need for culture of organism, possibility of direct application in clinical samples, lower biohazard risk and the feasibility of automation. However, not all mechanisms of drug resistance are known. Genotypic methods for drug resistance look for the genetic determinants of resistance rather than the

resistance phenotype and involve two basic steps – nucleic acid amplification by polymerase chain reaction (PCR) to amplify the sections of the *M.tuberculosis* genome known to be altered in resistant strains and a second step of assessing the amplified products for specific mutations correlating with drug resistance (Garcia de Viedma, 2003; Palomino, 2005). Nearly all RIF resistant strains contain mutation of the *rpoB* gene while mutations in the *embB* gene were associated with ethambutol (EMB) resistance (Cole, 1996; Riccardi et al, 2009). Mutations in *katG* gene and *inhA* gene were related to the high level and low level isoniazid (INH) resistance respectively (Vilcheze and Jacob, 2007).

The nature and frequency of mutations in the resistant strains vary significantly based on the geographical location (Mokrousov et al, 2002). There is very less information available on specific mutational patterns in India (Sharma et al, 2003), let alone on the underdeveloped and isolated region of northeast India. MDR TB strains have been reported mostly from countries where HIV and TB co-infection is endemic which includes India (Prasad, 2005). The dual challenges to TB and HIV co-infections are particularly pressing in Manipur, a state in the easternmost corner of northeast India which has the highest estimated adult HIV prevalence in India (NACO India, 2012)

The present study is being undertaken with an aim to extend the knowledge of mutational pattern of *M.tuberculosis* complex by using the MTBDR*plus* and MTBDR*sl* (Hain Lifescience) in Manipur, a geographically isolated region of northeast India. The study also evaluates the MTBDR method by comparing with phenotypic culture based method (BacT/Alert 3D) on an attempt to highlight the feasibility of applications in the current situation of TB, its drug resistance and diagnostic trends in northeastern India.

II. Material And Methods

Study settings.

This study was conducted at the Babina Diagnostic Centre in Imphal, Manipur which is one of the most prominent and main referral diagnostic lab of the region. It currently serves a population of 2.7 million. Testing was performed on residual portions of routine clinical specimens submitted for culture and DST. Informed consent was not taken for the study as results were unlinked from patient identifiers and no patient information was collected. Only one sample per patient was collected.

Sputum specimens

Three hundred and seventy five sputum samples were collected between May – December 2011. Every handling of the clinical specimens was conducted inside a Class II safety cabinet in aBSL2 laboratory in accordance with CDC guidelines. Sputum decontaminations were carried out with the conventional N-acetyl-L-cysteine-NaOH. After centrifugation, the pellet was suspended in 1ml of 85% NaCl. All specimens were processed for acid fast microscopy using Ziel-Neelsen technique (Canetti et al, 1963; Kent & Kubica, 1985). Sputum samples showing more than 10 acid fast bacilli (AFB) per microscopic field in the smear were selected for the study.

BacT/Alert 3D culture and DST

Drug susceptibility testing for isoniazid, rifampicin and ethambutol was performed by BacT/Alert 3D system. A 0.5ml portion of the sediment was inoculated into the vials of BacT/Alert 3D system containing modified Middlebrook 7H9 media with respective antibiotic. The final drug concentration in the test bottles was 1µg/ml for INH and RIF and 21µg/ml for EMB. All mycobacterial cultures were incubated at 37°C and were continuously monitored. *M.tuberculosis* isolate was determined to be resistant to the antibiotic when the drug containing bottle had a time to detection (TTD) that was less than or equal to the TTD of 1% control.

Genotype MTBDR*plus* and MTBDR*sl* DST

The MTBDR*plus* and MTBDR*sl* DST were performed according to the manufacturer's instruction and are divided into three steps: DNA extraction, a multiple amplification using biotinylated primers and reverse hybridisation. A 500µl portion of the decontaminated sediment was used for DNA extraction using modified CTAB-NaCl method. The isolated DNA samples from the process mentioned above were used for multiplex amplification with biotinylated primers. The biotin labelled PCR product was chemically denatured and hybridised to the strip with specific oligonucleotide probes. After hybridisation and washing, strips were removed, allowed to dry and fixed on the nitrocellulose paper.

All samples that tested positive for MDR in the MTBDR*plus* strip was further subjected to MTBDR*sl* test. Phenotypic DST was conducted for EMB only in the MDR strains which has been subjected MTBDR*sl*. All tests were performed independent of culture and DST.

Interpretation of results

Each strip in MTBDR*plus* consist of 27 reaction zones (bands) including six controls (conjugate, amplification, *M.tuberculosis* complex, *rpoB*, *katG* and *inhA* controls), eight *rpoB* wild type (WT) and four mutant (MUT) probes, one *katG* wild type and two mutant and two *inhA* wild type and four mutant probes

(Figure 1a). Each strip in the MTBDR_{sl} consists of 22 reaction zones including six controls (conjugate, amplification, *M.tuberculosis* complex, *gyrA*, *rrs* and *embB* controls). Three *gyrA* wild type and six mutant probes, two *rrs* wild type and two mutant probes and one *embB* wild type and two mutant probes (Figure 1b). Results were interpreted according to the manufacturer's instructions. Statistical methods were performed using SPSS 17.0. Results were considered significant at P value less than 0.5

III. Results

Genotype MTBDR_{plus} testing from smear positive sputum

Genotype MTBDR_{plus} test results in comparison with BacT/Alert 3D DST of all smear positive specimens tested (n = 375) are summarised in Table 1. Fifteen specimens (4%) were culture negative and therefore no phenotypic DST results were available. Of these 15 culture negative specimens, 14 (93.33%) gave interpretable results by the MTBDR_{plus} method. Of the specimens with conventional DST results, 60(16.67%) were NDR, 20(5.6%) were RIF mono-resistant, 14(4.44%) were INH mono-resistant and 366(73.33%) were RIF and INH susceptible. In comparison with BacT/Alert culture and DST results (96%), a higher proportion of MTBDR_{plus} results (97.1%) were interpretable. This genotypic method detected 58 (97%) MDR, 1 RIF mono-resistant with one uninterpretable result out of the 60 MDR strains detected by phenotypic DST. The results for RIF mono-resistant was concordant with the phenotypic DST except for one strain which was detected as RIF and INH susceptible out of the 20 RIF mono-resistant by phenotypic DST. MTBDR_{plus} detected 269 susceptible strains including 13 culture negative strains while BacT/Alert system could detect 264 susceptible strains including 9 uninterpretable strains of MTBDR_{plus}. The sensitivity, specificity, accuracy, positive predictive values (PPV) and negative predictive values (NPV) are given in Table 2.

BacT/Alert culture and DST for smear positive specimens had a total turnaround time of 8 -12 days. For MTBDR_{plus} testing, the test took 1 – 2 days for smear positive results while MTBDR_{sl} testing of the MDR strains took another 2 days.

Table 3 shows the distribution of different banding patterns in drug resistant isolates, including MDR, INH mono-resistant and RIF mono-resistant strains while Table 4 shows the banding pattern of MDR isolates which has been subjected to MTBDR_{sl} test. Typical banding patterns obtained on MTBDR_{plus} and MTBDR_{sl} strips are shown in Figure 1.

The RIF resistant isolates displayed different mutations. The most common mutation was in the S531L region (MUT3) with 45.6% of all RIF resistant strains (55% of MDR and 19% of RIF mono-resistant strains) having the mutation in the *katG* codon with amino acid change of Ser-315-Thr1. This difference in prevalence of the S531L mutation between MDR and RIF mono-resistant strain was significant (P = 0.012). Two MDR strain had a D516V mutation (3.4% overall). Of the isolates with mutation in the wild type probes, mutation in the WT8 was most common, as detected by the lack of binding to the WT8 probe in the absence of S531L mutation except for one strain which had both the WT8 wildtype and the S531L mutation. No mutation was detected in the MUT2A andMUT2B band in both the RIF mono-resistant and MDR strains. There was no significant difference in the presence of other bands between MDR and RIF mono-resistant strains. Of all the INH resistant strains, 73% (71% of MDR and 81% of INH mono-resistant strains) had a mutation in the *katG* S315T1 region while 4% had a mutation in the S315T2 region indicating high level resistance while 19%(19% Of MDR and 19% of INH mono-resistant strains) had mutation in the C15T region of *inhA* gene indicating low level resistance. This difference in prevalence of mutations in MDR strains compared with INH mono-resistant strains was significant for *katG* (P = 0.0073) but not for *inhA* (P = 0.1497). Five strains had mutations in both the *katG* and *inhA* genes. Seventy six percent (28/37) of MDR strains had a mutation in the *katG* gene and were detected as INH resistant y a mutation in the *katG* gene. Only three of 16 (18.8%) INH mono-resistant strains were detected by the presence of a mutation in *inhA* only

Genotype MTBDR_{sl} testing from MDR positive samples.

All samples tested for XDR by MTBDR_{sl} were fortunately negative which indicates that XDR is not yet detected from this region. This non-detection of XDR was all due to no mutation being detected in the *rrs* gene probe while mutation was detected in all the mutation region of *gyrA* gene with the highest rate in the D94G region (17%). One MDR positive strain had a *gyrA* MUT3D mutation. This is a rare mutation which has been detected only *in silico* and hence thought to be undetectable *in vitro*. But this detection albeit in one strip proves that it is detectable and that the MTBDR_{sl} is efficient in detecting even rare mutations. Of the 32 EMB resistant strains according to the molecular method, 5 strains had mutations in the *embB* gene region of codon 306 with amino acid change of Met-306-Ile and 27 strain had mutation in the *embB* gene with amino acid change of Met-306-Val. One strain had mutation that was detected only at the wild type probes (*embB* WT) but not at the mutant probes.

Performance of Genotype MTBDR_{plus} and Genotype MTBDR_{sl} assays

Performance parameters for detection of INH/RIF mono-resistant, MDR and EMB resistance were calculated from specimens for which genotypic and phenotypic DST results were available (Table 2). The performance of the genotypic MTBDR*plus* test directly from smear positive sputum correlated highly with the phenotypic BacT/Alert automated DST. Compared with the phenotypic DST, the genotypic MTBDR*plus* had a sensitivity of 81.3%, specificity of 99.2% and accuracy of 98.2% for detection of INH, a sensitivity of 95%, specificity of 100% and accuracy of 99.6% for RIF resistance and a sensitivity of 98.3%, specificity of 100% and accuracy of 99.7% for MDR. The genotypic MTBDR*sl* assay for ethambutol had a sensitivity of 86%, specificity of 91% and accuracy of 88% for detection of EMB resistance.

IV. Discussion

The efficiency of Genotype MTBDR have been reported by many authors (Hilleman et al, 2006, 2007; Barnard et al, 2007, 2008) having good concordance with phenotypic DST results but presence of false negative due to unique mutation are also reported (Hilleman et al, 2007; Brossier et al, 2006; Tessema et al 2012)). This may be due to specific location independently developing endemic mutations. The present study evaluates the INH, RIF, MDR and EMB mutations using genotypic MTBDR*plus* and MTBDR*sl* test compared with BacT/Alert 3D DST of a geographically isolated region of northeast India. The results of the study showed that the mutational pattern of the resistant genes didn't vary much with most mutations confined to one or two main regions. It is seen that in this region of India, the most frequent mutation causing RIF resistance is the Ser-531-Leu mutation (45.6%) and in INH resistance, the Ser-315-Thr1 is the most common (19%). This is more or less in agreement with earlier reported studies (Miotto et al, 2006; Mokrousov 2002). In case of EMB resistance, Met-306-Val mutation (45%) is observed to be the major cause. Previous studies have shown that 40-95% of isoniazid resistance are defined as the high level drug resistance due to *katG* gene mutations. In this present study, 77% of INH resistance are attributed to *katG* mutations of which 95% are S315T1 and 5% are S315T2 mutations. A high prevalence of *katG* mutations has been reported to account for a high proportion of INH resistance in high TB prevalent countries presumably due to ongoing transmission of these strains in high burden settings (Mokrousov et al 2002). India, being one of the top high TB burden countries, such high prevalence was expected.

The performance characteristics of specificity, sensitivity, accuracy, PPV and NPV as given in Table 2 suggest that the MTBDR*plus* assay is equivalent to phenotypic culture based DST performed in quality assured reference laboratories but detection of ethambutol resistance by MTBDR*sl*, though lower than INH or RIF detection by MTBDR*plus*, is comparatively higher than other reported studies (Brossier et al 2010; Tesema et al 2012). The lower value of sensitivity for detection of INH resistance (81.25%) by genotypic method might be due to resistance inferred by mutations in other genomic region which has not been incorporated in this assay, such as *ndh* and *kasA* gene (Lee et al, 1999, 2001; Rindi et al, 2005). Three out of 16 INH mono-resistant strains were detected by mutations in *inhA* gene only. These mutations would not have been detected by the previous MTBDR which didn't incorporate the *inhA* probe. As such, mutations in other genomic regions might be inducing resistance to INH which would not be detected by genotypic method if their corresponding probes were not included, leading to false positives apart from the false negative results due to presence of inhibitors (Palomino, 2006).

Identification and drug resistance assay of *M. tuberculosis* normally requires several weeks as they are very slow in their growth and even with automated culture system, it takes an average of 14 days. Another 14 days for additional tests are required for DST. Moreover, the culture based methods involve increased consumption of exclusive culture media and hence place more economic burden on patients especially in a low resource and high burden region like Manipur. On the basis of FIND-negotiated prices, the cost of molecular assay is less than 50% of that for conventional liquid culture and DST for INH and RIF (Barnard et al, 2008) Therefore there is urgency for an efficient method of TB testing as a complement to conventional culture and smear microscopy.

In conclusion, the results of this study supports the use of MTBDR*plus* for rapid diagnosis of TB considering the drastic reduction of time in diagnosis and its accuracy being comparable to conventional methods. It might also be more cost effective in the long run. This molecular genotyping will also be useful in studying epidemiological and mutational analysis of *M. tuberculosis* of specific regions.

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Table 1. Results of MDR by Genotype MTBDRplus in comparison with BactT/Alert culture and DST.

Genotype MTBDRplus	BactT/Alert 3D and DST				
	MDR	RIF mono-resistant	INH mono-resistant	RIF & INH susceptible	Culture negative
MDR	58	0	0	0	0
RIF mono-resistant	1	19	0	1	0
INH mono-resistant	0	0	13	2	1
RIF & INH susceptible	0	1	3	252	13
Uninterpretable	1	0	0	9	1

Definition of abbreviation: DST = Drug Susceptibility Test; RIF = Rifampicin; INH = Isoniazid; MDR = Multidrug resistant

Table 2. Performance of MTBDRplus in detecting MDR from smear positive sputum specimens

Parameters	RIF	INH	MDR	EMB
Sensitivity	95.0(93.0 – 97.0)	81.25(78.0 – 85.0)	98.3(97.2 – 99.5)	85.71(82.6 – 88.8)
Specificity	100 (99.0 – 100)	99.21(98.4 – 99.0)	100 (99.0 – 100)	91.3 (88.8 – 93.8)
Accuracy	99.63(99.1 – 100)	98.15(97.0 – 99.3)	99.63(99.1 – 100)	87.93 (85.0 – 90.8)
PPV	100 (99.0 – 100)	86.677(84.0 – 90.0)	100 (99.0 – 100)	93.75(91.6 – 995.9)
NPV	99.6 (99.1 – 100)	98.82(97.0 – 99.3)	99.6 (99.0 – 100)	80.77(85.0 – 90.8)

Definition of abbreviations: NPV = negative predictive value; PPV = positive predictive value. Values are percentages with 95% confidence interval in parentheses.

Table 3. Pattern of gene mutations in resistant *M. tuberculosis* strains using Genotype MTBDRplus assay

Gene	Band	Gene region of mutation	MDR (n = 58)	INH Monoresistant (n = 16)	RIF Monoresistant (n = 21)
rpoB	WT1	505-509	54(93)	16(100)	18(86)
	WT2	510-513	54(93)	16(100)	21(100)
	WT3	513-517	49(84)	16(100)	19(91)
	WT4	516-519	54(93)	16(100)	19(91)
	WT5	518-522	56(97)	16(100)	19(91)
	WT6	521-525	49(84)	16(100)	13(62)
	WT7	526-529	49(84)	16(100)	12(57)
	WT8	530-533	13(22)	16(100)	0(0)
	MUT1	D516V	2(3)	0(0)	0(0)
	MUT2A	H526Y	0(0)	0(0)	0(0)
	MUT2B	H526D	0(0)	0(0)	0(0)
	MUT3	S531L	32 (55)	0(0)	4(19)
	katG	WT	315	13(22)	2(13)
MUT1		S315T1	41(71)	13(81)	0(0)
MUT2		S315T2	2(3)	1(6)	0(0)
inhA	WT1	-15/-16	47(81)	13(81)	21(100)
	WT2	-8	55(95)	15(94)	21(100)
	MUT1	C15T	11(19)	3(19)	0(0)
	MUT2	A16G	0(0)	0(0)	0(0)
	MUT3A	T8C	0(0)	0(0)	0(0)
	MUT3B	T8A	0(0)	0(0)	0(0)

Definition of abbreviations: RIF = rifampicin; INH = isoniazid; MDR = multidrug resistant; Values are numbers, with percentages in parentheses.

Table 4. Pattern of gene mutations in MDR *M. tuberculosis* strains using Genotype MTBDRsl assay

Gene	Band	Gene region of mutation	Pattern of mutation (n = 58)
gyrA	WT1	85-90	58(100)
	WT2	89-93	51(88)
	WT3	92-97	44(75)
	MUT1	A90V	8(14)
	MUT2	S91P	3(5)
	MUT3A	D94A	5(9)
	MUT3B	D94N/Y	5(9)
	MUT3C	D94G	10(17)
	MUT3D	D94H	1(2)
	rss	WT1	1401-1402
WT2		1484	24(100)
MUT1		A1401G/C1402T	0(0)
MUT2		G1484T	0(0)
embB	WT1	306	25(43)
	MUT1A	M306I	5(9)
	MUT1B	M306V	27(47)

Values are numbers, with percentages in parentheses.

Figure 1.

1a. Examples of GenoTypeMTDBR^{plus} strips (Hain Lifescience, Nehren, Germany). (Lane 1) Multidrug-resistant tuberculosis (MDR TB), *rpoB* S531L mutation and *inhA* C15T mutations. (Lane 2) MDR TB, *rpoB* S531L mutation and *katG* S315T1 mutation. (Lane 3) *M. tuberculosis*, INH monoresistant (*katG* S315T1 mutation). (Lane 4) *Mycobacterium tuberculosis*, RIF monoresistant (*rpoB* S531L mutation in 530–533 region). (Lane 5) *M. tuberculosis* susceptible to isoniazid (INH) and rifampin (RIF). (Lane 6) Negative control.

1b. Examples of GenoType MTBDR^{sl} (Lane 1) MDR Tb with *gyrA* WT3/ D94G and *embB* M306V mutation (Lane 2) *gyrA* WT2/S91P and *embB* M306I mutation (Lane 3) *gyrA* WT3/D94NY/D94G and *embB* M306V mutation (Lane 4) MDR TB susceptible to Flouroquinone, Cyclic peptides and Ethambutol (Lane 5) Negative control.

