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Diagnostic Accuracy of Line Probe Assay for Detecting Multidrug Resistant Tuberculosis in Clinical Specimens

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Abstract

Setting: The study was carried out at Microbiology Department, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from June 2013 through March 2015.

Rapid detection of drug resistance is a priority area in tuberculosis (TB) research. GenoType MTBDR*plus* is a commercially available line probe assay (LPA) simultaneously detecting mutations in the *inhA*, *katG*, and *rpoB* genes that confer isoniazid (INH) and rifampicin (RIF) resistance. The objective of the study was to evaluate the diagnostic accuracy of GenoType MTBDR*plus* for detecting multidrug resistant (MDR) TB in smear positive clinical samples.

Design: Cross sectional validation study.

Methodology: 186 samples from 186 patients containing acid fast bacilli (AFB) were decontaminated and subjected to GenoType MTBDR*plus* and simultaneously processed for culture and first line anti-TB drugs sensitivity on MGIT 960 system.

Results: Among 95 MDR TB isolates, GenoType MTBDR*plus* detected 92 isolates with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and the diagnostic accuracy of 97%, 96%, 96.5, 98% and 97% respectively.

Conclusion: Diagnostic accuracy of 97% and turnaround time of 5 hours makes it a promising tool when compared with conventional method MGIT 960 for detection of drug resistant tuberculosis in resource limited countries.

Keywords: *MGIT: Mycobacterium growth indicator tubes; AFB: Acid fast bacilli; Mutation probes; Hetero resistance*

Introduction

Drug resistant TB remains a persistent threat to global public health. About 480,000 reported cases of MDR TB occurred globally in 2013 [1]. Treatment of MDR TB involves prolong use of second line anti-TB drugs having low efficacy tolerance, more toxicity and cost [2]. The dilemma can be addressed with early diagnosis aiming prompt treatment. World Health organization (WHO) recommends molecular-based assays for detecting drug resistance [2]. Rapid diagnosis of MDR TB is pivotal to avoid inaccurate treatment.

GenoType MTBDR*plus* is a commercially available LPA that detects mutations in the *inhA, katG* and *rpoB* genes which confer INH and RIF resistance [3,4]. The assay combines detection of *Mycobacterium tuberculosis* (MTB) complex with mutations in the 81 base pair hotspot region of *rpoB*, at codon 315 of the *katG* gene and in the *inhA* promoter region [5,6]. The assay has proved efficacious in the developed countries [7,8]. However, validation of this rapid tests in TB endemic settings like ours yet is to be evaluated. Thus this study was conducted to determine the diagnostic accuracy of GenoType MTBDR*plus* a molecular technique in our region by comparing it with the results obtained through conventional BACTEC MGIT 960 system.

Materials and Methods

This cross-sectional validation study was carried out at Microbiology Department, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from June 2013 through June 2015. Formal permission was sorted from Institutional Ethical Committee; consent was taken from all the patients.

Smear positive specimens by Ziehl-Neelsen (ZN) staining method were included in the study. Samples mainly comprised of sputum, pus, bronco-alveolar lavage, lung biopsy tissue, pleural aspirate, peritoneal aspirate and intestinal tissue biopsy samples. Repeat samples, in adequate samples and contaminated samples were excluded from the study.

The specimens were processed as per the manufacturer's instructions [9]. Specimen homogenization and decontamination was done by using standard sodium hydroxide-N-acetyl-L-cysteine method using 2% NaOH. Direct smear was prepared from the processed sediment and stained by ZN staining and a record of AFB index was kept [10]. Each specimen was processed simultaneously on MGIT 960 system (BD BACTECTM) and GenoType MTBDR*plus* (HAIN® Lifescience).

The GenoType MTBDR*plus* assay testing was performed according to the manufacturer's guideleines [9]. Briefly, the test involved: DNA extraction, multiplex polymerase (PCR) amplification and reverse hybridization. Each strip consisted of 27 reaction zones or bands including 6 controls (conjugate, amplification, *M. tuberculosis complex, rpoB, katG* and *inhA*) 8 rpoB wild types (WT), 4 mutant (MUT) probes, 1 katG wild type and 2 mutant probes and 2 inhA wild types and 4 mutant probes. The results obtained on the strips were recorded in a turnaround time of 5 hours [10]. Quality control was ensured at each step. *Mycobacterium tuberculosis* ATCC 27294 was used as control strain.

MGIT 960 tubes were incubated for a period of six weeks before reporting as negative. Three MGIT tubes were supplemented with 0.5 ml of OADC (oleic acid, bovine albumin, dextrose, and catalase), 0.1 ml of PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin), and 0.1 ml of test antibiotic; the third tube, being the growth control (GC), was without antibiotic. The lyophilized antibiotics were provided by and prepared as per recommendations by the manufacturer (BACTEC MGIT 960 SIRE kit, Becton Dickinson, Baltimore, MD). The final concentrations in the test tubes were INH at 0.1µg/ml and RIF at 1.0µg/ml. Equal volumes (0.5 ml) of the processed specimen were inoculated into the three tubes and then incubated at 37°C. To exclude bacterial contamination, an aliquot of the processed specimen was also inoculated onto a sheep blood agar plate, incubated at 37°C, and examined after 48 h.

On day 3, tubes were examined daily using a 365-nm UV transilluminator and their fluorescence levels were compared with negative and positive control tubes; the negative control was an un-inoculated tube, and the positive control was an MGIT tube containing MTB ATCC 27294. An isolate was considered susceptible to the test drug if the drug-containing tube did not fluoresce within 2 days of the GC tube fluorescing. Conversely, an isolate was defined as resistant if the drug-containing tube fluoresced within 2 days of the GC tube.

Data was analyzed on SPSS version 17. Mean and standard deviation were calculated for all quantitative data. Percentages were calculated for all qualitative data. Sensitivity, specificity, PPV, NPV and diagnostic accuracy of GenoType MTBDR Plus for multidrug resistant tuberculosis were calculated.

Results

A total of 186 samples were dealt with. Mean age of 186 patients included in the study was 44 ± 3.24 years; a range 30 to 60 years. Out of 92 MDR isolates detected by GenoType MTBDRplus, maximum cases 75(81%) were in the age group of 30-60 years.

Male to female ratio was 3:1. Specimens included sputum 119(64%), bronco-alveolar lavage 35(19%), pus 22(12%) and fluids 5(3%) like pleural aspirate and peritoneal aspirate.

According to the AFB index scale on ZN staining, 19(10%) were 1+, 23(12%) were 2+, 114(61%) were 3+and 30(16%) were 4+. Among 186 samples, Genotype MTB DRplus detected 92 samples as MDR TB while MGIT 960 detected 95 MDR TB. Considering AFB index of 186 samples, 114(61%) of the specimens were detected to be 3 plus category. Among 92 MDR specimens as detected by GenoType MTBDR*plus*, 6(3.22%), 12(6.4%), 45(24%) and 29(15%) having 1+, 2+, 3+and 4+ respectively on AFB index scale as in table 1.

89 (47%) were true positives and 92(50%) samples true negatives. 3(1.6%) were false positives and 2(1.07) false negatives. Genotype MTBDR*plus* failed to detect only 2(1%) of MDR samples. Sensitivity, specificity, positive predictive value, negative predictive value of Genotype MTBDR*plus* was 97%,96.1%, 96.5%, 98% respectively while diagnostic accuracy was 97%. In our study, 2(1.06%) specimens exhibited hetero resistance which means positive hybridization showing mutant and wild type probes simultaneously. The site and frequencies of specific mutations as detected by GenoType MTBDR*plus* for RIF (*rpoB*) and INH (*Kat G, Inh A*) are shown in table 2.

Discussion

MDR TB is on the rise more so in developing countries [11,12]. In Pakistan TB prevalence is 263/100,000 with 3.4%.annual rate of new cases of MDR TB [13]. The ground reality is far worse due to lack of diagnostic facilities. This necessitates rapid and accurate diagnostic methods to address this serious threat. WHO took special initiative to make LPA accessible and affordable for resource limited countries [13,14]. WHO aims to reduce the TB burden to the earliest possible. This idea seems implausible in the current scenario, especially in areas with increasing MDR TB like in the country where MDR TB in new cases (3.4%) is mostly under diagnosed while retreatment cases (35%) are increasing gradually [13].

In resource limited countries like ours, conventional culture on Lowenstein Jenson (LJ) medium is still used in most of the laboratories. Rapid diagnostic facilities are available in very few tertiary care centers. Molecular diagnostic tools like Gene Xpert and GenoType MTBDR*plus* are scarce in a country which stands fifth among the highest MDR TB burden countries across the globe [13].

Results of some recent studies demonstrated that MDRTB *plus* assay can be used as an effective diagnostic modality for MDR TB screening [14]. In our study, we selected smear positive specimens to see the diagnostic efficacy of GenoType MDRTB *plus* system [15,16].

Sputum was the most common specimen received 105 (56%) followed by endobronchial washings 44 (23%) and pus 29(15%). Genotype MTBDR*plus* correlated well with AFB index in our study. Among 92 MDR specimens, 6(3.22%), 12(6.4%), 45(24%) and 29(15%) having 1+, 2+, 3+and 4+ respectively on AFB index scale. There is significant directly proportional relationship between the number of MDR cases and increasing ABF positivity as seen in a previous regional study in 2010 [17]. All the cases with highest AFB positivity 3+ were equally picked by GenoType MTBDRplus [18].

In our study, on the whole results of GenoType MTBDR*plus* matched the gold standard MGIT 960 and this was comparable to a similar study conducted in another high TB burden region [19]. Out of 186 isolates, 92 were found MDR TB by GenoType MTBDR*plus* while 94 isolates were non MDR/MTB, having no or mono resistance to either of the first line anti TB drugs. In186 isolates, resistance seen for RIF and INH was 102 (54%) and 112 (60.1%) respectively.

In our study 2(1%) MDR isolates were missed by GenoType MTBDR*plus* when compared with BACTEC MGIT 960.The probes can cater to detect common mutations in our region but the need to expand the number of probes for common regional mutations will improve its sensitivity as mentioned in a study by Farooq *et al.* earlier [14].

The sensitivity of the assay gets affected by optimal selection of DNA probes and targets for the variable population studies because in different studies the prevalence of mutations associated with *rpoB* and *inhA* resistance varies in different geographical regions. The mutations associated with the resistance to RIF, maximum mutations were in the codon 531-533 of the *rpoB* gene with specific mutation of S531L. As shown in table 2, among wild type deletions, wild type: WT8 (codon 531-533) 74(69%) was the commonest of the deletions and higher than the percentage of mutation in another local study (67%) [14]. However, combined deletion patterns 7(6.5%) were also observed as WT3, 4 (codon 513-6,516-8) .Slightly lower than a previous regional study (11%) [14]. 41 (38.3%) mutations were MUT 3(S531L) in rpoB gene. S531L is another hotspot apart from 531-533 where mutations are observed frequently in our region [18]. In resistance against INH, our study shows increased rates of wild type deletions and mutations when compared to a regional study by Farooq *et al*. [19]. MDR isolates had missing wild type WT at mutation site 315 was 86(69%) and 76(61%) mutations MUT 1 occurred in codon S315T of the *katG* gene. S315T(ACG-ACC) region was found to be the most frequently mutated region of *katG* gene conferring to high level resistance against INH. 8(6.41%) deletions of wild types WT1at -15,-16 contributed to the low level resistance for INH (*inhA*)

as seen in table 2. In general, it was similar to previously reported Russian study conducted on a larger population [18]. In another study from Pakistan, similar mutations were observed, however our study showed variable percentages specially mutations in regions 531-533, S531L,315, S315T(ACG-ACC) as shown in table 2. This signified the changing trends of mutations within a region [20,21].

A recent meta-analysis showed that line probe assay had excellent accuracy for RIF resistance, even when directly used on clinical specimens [22], specificity was excellent for INH with variable sensitivity [14,15]. Genotype MTB DR Plus is equally good for detecting both RIF and INH resistance individually as the sensitivity and specificity of Genotype MTB DR Plus is beyond 92% for both the first line drugs RIF and INH, this observation is comparable to the regional studies [14]. The sensitivity of the method in our study was comparable to various studies conducted in South Africa (96%), Italy (98), Russia (97%) and Germany (97%) [19,20]. Selecting and expanding the number of probes on DNA strip according to regional spectrum can further enhance the sensitivity of this useful tool. Specificity and PPV achieved by this method was comparable to a study conducted in Vietnam by Hyuen *et al* where the Specificity is 100% [4]. However, the negative predictive value was 96.8%.

Heteroresistance is an important phenomenon which means positive hybridization with mutant and wild type probes or double patterns which simply confuse and effect the interpretation. It can be taken as an alarm for potential MDR cases as the wild type patterns are still intact and mutations have occurred. In other words this is the stage when resistance can be limited by adequate treatment and containment measures. This pattern was first reported by Rinder *et al*. [22]. In our study 2 (1.07%) of the samples showed hetero resistance; the phenomenon has been observed in previous studies [14,21,22].

With 97% diagnostic accuracy, this tool can serve as one of the efficient and handy diagnostic methods to achieve the bench mark of WHO. In recent years, incidence of extensively drug resistant TB and even total drug resistance tuberculosis is also increasing, gradually dragging us back to the pre antibiotic era [22,23]. Early detection of MDR-TB and XDR-TB is critical to initiate appropriate treatment, reduce morbidity and mortality, and prevent further transmission of drug-resistant strains of mycobacterium tuberculosis.

We recommend that the Genotype MTBDR*plus* test should serve as an early guidance for therapy, which should be followed by a phenotypic DST confirmation for all suspected MDR TB patients. Incorporation of this molecular test in the National Tuberculosis Program will be an important step forward in not only the rapid diagnosis of MDR TB among suspected patients but also to keep a track of ongoing regional mutations. This will help pulmonologists to steer the treatment regimens effectively.

Conclusion

GenoType MTBDR*plus* test is a reliable, rapid and easy method with simultaneous detection of RIF and INH resistance in *M. tuberculosis*. With fairly high sensitivity and specificity for detecting MDR TB in smear positive specimens, this test strongly facilitates in the commencement of right and timely treatment of the MDR-TB patients much before results of conventional tests are available, especially important for developing countries like Pakistan.

AFB Index	AFB Index of Samples n (%)	MGIT 960 System n (%)	GenoType MTBDRplus n (%)
+	19(10.3)	7(3.76)	6(3.22)
++	23(12.36)	13(6.98)	12 (6.45)
+++	114(61.2)	45(24)	45(24)
++++	30(16)	30(16.12)	29(15.59)

Table 1: *Percentage of AFB positivity in MDR TB cases as picked by conventional and the molecular systems.*

Probes	Mutation Sites	n (%)	Specific Mutations	n (%)
rpoB				
WT8	531,533	74(69)		
MUT3			S531L	41(38.3)
WT3,4	513-6,516-8	7(6.5)		
MUT1			D516V	3(2.8)
WT2,3	510-3,513-6	4(3.7)		
WT7	526	1(0.9)		
MUT2A			H526Y	2(1.8)
katG				
WT	315	86(69)		
MUT1			S315T(ACG-ACC)	76(61)
MUT2			S315T(ACG-AAC)	2(1.6)
inhA				
WT1	-15,-16	8(6.4)		

Table 2: *Frequencies of mutations as detected by GenoType MTBDR Plus.*

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