Original Article

Comparative Efficacy of Line Probe Assay with Conventional Culture for Detection of Drug-Resistant *Mycobacterium tuberculosis*

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Background: Multidrug-resistant tuberculosis [MDR-TB] has emerged as an important global public health threat. As gold standard for drug-resistant tuberculosis diagnosis, TB culture and drug susceptibility testing [DST] results still take several weeks. A new molecular diagnostic technique, or commercial line probe assays [LPA], has been developed for rapid detection of drug-resistant TB.

Objective: To compare the efficacy of a LPA for rapid detection of rifampicin [RIF] and isoniazid [INH] resistant tuberculosis in clinical specimens with conventional method.

Materials and Methods: The LPA (GenoType MTBDR*plus* assay) was performed directly on 54 consecutive smear positive specimens at the clinical microbiology laboratory of Bamrasnaradura Infectious Diseases Institute [BIDI], Thailand between January 2014 and December 2014 for the detection of RIF and INH resistance TB. Results were compared with conventional liquid culture and DST.

Results: Overall concordance of RIF and INH susceptibility results between LPA and conventional culture with DST was 87.0% and 92.6%, respectively. Compared with conventional method, the sensitivity and specificity were subsequently 100% and 86.5% for the detection of RIF resistance; whereas, 100% and 92.1%, respectively, for the detection of INH resistance by LPA.

Conclusion: LPA is highly sensitive for diagnosis and detection of drug-resistant tuberculosis, with more diagnostic accuracy in INH resistance than RIF resistance when performed directly on smear positive specimens.

Keywords: Line probe assay, Conventional culture, Drug resistance, Tuberculosis

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Multidrug-resistant tuberculosis [MDR-TB], defined as resistance to at least isoniazid [INH] and rifampicin [RIF], is one of the main health problems, particularly in developing countries including Thailand. According to the World Health Organization [WHO] report[1], an estimated 480,000 people developed MDR-TB worldwide in 2014, and there were an estimated 190,000 deaths from MDR-TB. Data from drug resistance surveys and continuous surveillance among notified TB cases suggest that 3.5% of newly diagnosed TB cases, and 21% of those previously treated for TB had MDR-TB[2]. Diagnostic delays often result in missed or late diagnosis with serious consequences of TB patients[3] .

Timing of diagnosis and treatment of MDR-TB is crucial in curtailing the spread of the infection in the community. Conventional drug susceptibility testing [DST] with solid culture has been the gold standard but takes up to 4 to 6 weeks after the growth of *Mycobacterium tuberculosis*. Although use of liquid media for drug susceptibility are more sensitive and faster than solid culture[4,5], liquids systems require stringent quality assurance systems, training standards and equipment investments. Commercial line probe assays [LPA] based on the detection of genetic mutation, have rapid turn-around time of 2 to 3 days and have been introduced in many countries. Two commercial LPAs are available: the INNO-LiPA RIF.TB (Innogenetics NV, Belgium) and GenoType MTBDR*plus* assay (Hain Life-science, Germany). The commercial strip assay INNO-LiPA RIF. TB has been evaluated for the detection of mutations conferring resistance to RIF in *M. tuberculosis*[6-8]. The Genotype MTBDR*plus* assay is a kit-based method for the detection of the most common mutations in *M. tuberculosis katG* and *rpoB*. In 2008, the WHO

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recommended the LPA as a rapid diagnostic tool to define drug susceptibility of *M. tuberculosis* in smear positive specimens or in isolates of this organism grown from smear negative specimens[9].

From a meta-analysis in 2008 found that GenoType MTBDR*plus* assay and another commercial test that a pooled sensitivity of 98% for detecting RIF resistance and 89% for detecting INH resistance and specificity of 99% for RIF and INH[10]. However, there is a wide variation in circulating *M. tuberculosis* strains across the world[11,12] and false negative results can occur due to the presence of genetic mutations in the different settings[13-18]. Therefore, validation in different settings is needed to ensure acceptable performance. One study in Thailand showed that the GenoType MDRTB*plus* assay had a sensitivity of 95.3%, 100% and 94.4% for detecting INH resistancer, RIF resistance and MDR-TB, respectively. Meanwhile specificity was 100% for all resistance pattern[19].

Due to the variation of *M. tuberculosis* strains in different settings, therefore, we compared sensitivity and specificity of LPA (GenoType MTBDR*plus* assay) in detecting RIF and INH-resistant strains from smear positive samples with the conventional culture and susceptibility method in BIDI to assess accuracy of the LPA.

Materials and Methods

The present study was conducted in the Department of Microbiology at Bamrasnaradura Infectious Diseases Institute [BIDI], Thailand. A total of 69 from 99 acid fast smear-positive clinical samples were tested with both LPA [GenoType MTBDR*plus* assay (Hain Life Science, Germany)] and conventional culture with DST between January and December 2014. Fifteen specimens were nontuberculous mycobacteria [NTM] excluded from the study. We retrospectively collected the results of the LPA that performed on 54 consecutive smear positive specimens (sputum = 44, lymph node aspiration = 4, pus from abscess = 3, cerebrospinal fluid [CSF] = 1, joint fluid = 1 and feces = 1) for the detection of RIF and INH resistance compared with conventional method (culture-based susceptibility testing).

Conventional culture and drug susceptibility testing

Clinical specimens were processed using N-acetyl-L-cysteine-sodium hydroxide [NALC-NaOH] method according to the CDC publication[20]. After decontamination, the concentrated samples were cultured on Lowenstein-Jensen [LJ] medium. The isolates of *Mycobacteria* were sent to the national TB reference laboratory [NTRL] for *M. tuberculosis* complex identification and DST of liquid-based cultures.

Genotype MTBDRplus assay

The Genotype MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 µl of AM-B (primer-nucleotide mix), 10 µl of AM-A (thermostable *Taq* DNA polymerase) provided in the kit and 5 µl of extracted chromosomal DNA solution in a final volume of 50 µl. The following amplification parameters were used: 15 minute of denaturation at 95 °C, followed by 20 cycles of 30 second at 95 °C and 2 minute at 65 °C, followed by 30 additional cycles of 25 second at 95 °C, 40 second at 50 °C, and 40 second at 70 °C, ending with a final extension step of 8 minute at 70 °C. Hybridization and detection were performed with a TwinCubator semiautomated washing and shaking device according to the manufacturer's instructions and using the reagents provided with the kit. Briefly, 20 µl of denaturation solution was mixed to 20 µl of amplified sample and incubated at room temperature for 5 minutes. One milliliter of prewarmed hybridization buffer was added before the membrane strips were placed and shaken in the hybridization solution for 30 minutes at 45 °C followed by stringent wash step for 15 minutes at 45 °C. After two washing steps, a colorimetric detection of the hybridized amplicons was obtained by the addition of the streptavidin alkaline phosphatase conjugate.

Interpretation of LPA results

An isolate was considered sensitive if all wild type probes tested positive and there was no hybridization with mutation detection probes. The absence of at least one wild type probe indicated resistance of the tested strain to the respective antituberculous drug. Hetero-resistance was defined when bands for both wild type and mutation probes were detected simultaneously in a specimen.

Data analysis

The sensitivity, specificity and accuracy of LPA results were compared to the conventional culture and DST results. Categorical variables were reported as number and percentage. Descriptive analysis was performed using SPSS version 14.0.

Ethical consideration

The present study was approved by the Institutional Review Board of the BIDI, Nonthaburi, Thailand. The study received a waiver of informed consent because the present study used samples from routine collection and test on media and molecular method.

Results

A total of 54 AFB smear-positive samples (44 samples from sputum) were tested with GenoType MTBDR *plus* assay, one of the LPA. Of these specimens, 41 (75.9%) were from male and 13 (24.1%) from female. Age ranged from 17 to 77 years (Mean 39.96±14.55 years).

Conventional culture and DST identified 2 (3.7%) and 3 (5.5%) of the 54 strains as resistant to RIF and INH, respectively (Table 1), while using the LPA, the corresponding resistance rates were 9 (16.7%) and 7 (12.96%). There were 7 and 4 strains identified as resistant to RIF and INH by LPA, but sensitive by conventional DST. The sensitivity and specificity for detection of RIF resistance by Genotype MTBDR*plus* assay was found to be 100% (95% CI: 15.81% to 100%) and 86.5% (95% CI: 74.2% to 94.4%), respectively. For INH resistance, sensitivity and specificity were 100% (95% CI: 29.2% to 100%) and 92.1% (95% CI: 81.1% to 97.8%) (Table 1). Overall concordance between Genotype MTBDR*plus* assay and conventional method results were found to be 87% and 92.6%, respectively.

From total of 54 specimens, 13 specimens (24.1%) identified as resistant to RIF and/or INH by conventional DST and/or LPA, discordant results were found in 8 samples. All of these specimens were not shown as RIF or INH resistance by conventional DST which is a gold standard method, but 4 samples were detected as MDR-TB, 3 strains as RIF-resistant *M. tuberculosis* and 1 strain as INH-resistant *M. tuberculosis* by LPA (Table 2).

The most common genetic mutation conferring RIF resistance was detected by missing of *rpo*B wild type band 8 (*rpo* BWT8), followed by loss of wild type band 3 and 4. Meanwhile, the common mutation detecting INH resistance was C15T in *inh*A gene and S315T1 in *kat*G gene, respectively (Table 3).

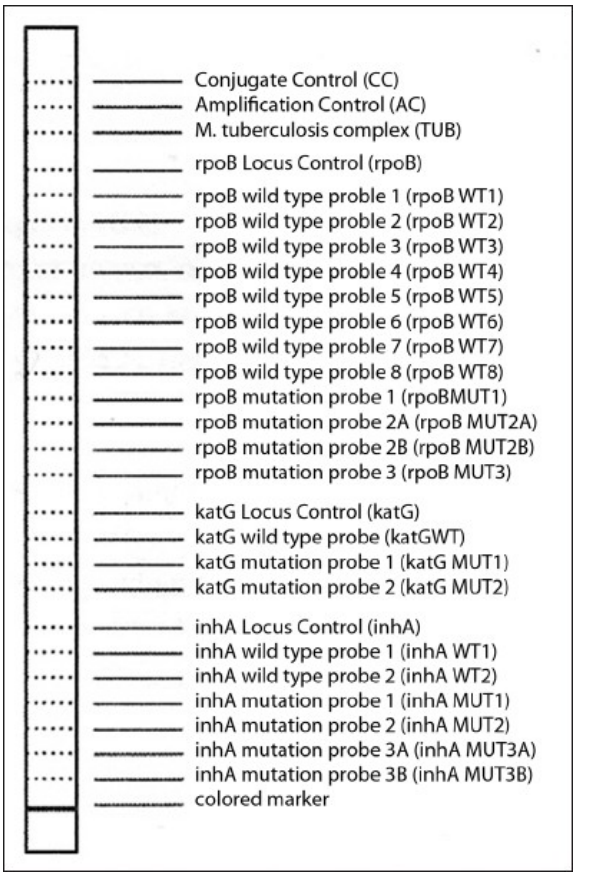

Figure 1. GenoType MTBDR*plus* assay strip. The strip is not displayed in original size

Discussion

The emergence of MDR-TB remains a serious threat to global TB control. Accurate and early diagnosis of MDR-TB is highly desirable as it interrupts transmission of the disease and avoids inadequate treatment regimens. LPA has been introduced for rapid molecular detection of drug resistance from smear-

Table 1. Comparison of LPA with Conventional culture for detection of rifampicin and isoniazid resistance (n = 54)

	Conventional culture and DST				
LPA	Resistant	Susceptible	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Accuracy (%)
RIF			100 (15.81-100%)	86.5(74.2-94.4%)	87
Resistant (rpoB)	2	7			
Susceptible	0	45			
INH			100 (29.2-100%)	92.1(81.1- 97.8%)	92.6
Resistant (KatG/inh A)	3	4			
Susceptible	0	47			

positive specimens. The GenoType MTBDR*plus* assay, one of the LPA, provides comprehensive information about the mutations leading to drug resistance which highly correlates with the sequencing results. The accuracy of the GenoType MTBDR*plus* assay from one metaanalysis had demonstrated the pooled sensitivity and specificity in detecting RIF resistance to be 98.1% (95% CI: 95.9 to 99.1) and 98.7% (95% CI: 97.3 to 99.4) higher than INH resistance 84.3% (95% CI: 76.6 to 89.8%) for sensitivity and 99.5% (95% CI: 97.5 to 99.9%) for specificity, but sensitivity estimates were highly heterogeneous across the studies. Results were similar across all assay and specimen types[10]. Nevertheless, little research has been conducted in Thailand.

Table 2. Discordant results between LPA and Conventional culture with drug susceptibility testing for detection of rifampicin and isoniazid resistance

	LPA		Conventional culture	
Number	Rifampicin	Isoniazid	Rifampicin	Isoniazid
1	Resistant	Resistant	Susceptible	Susceptible
2	Resistant	Susceptible	Susceptible	Susceptible
3	Resistant	Resistant	Susceptible	Susceptible
4	Susceptible	Resistant	Susceptible	Susceptible
5	Resistant	Susceptible	Susceptible	Susceptible
6	Resistant	Susceptible	Susceptible	Susceptible
7	Resistant	Resistant	Susceptible	Susceptible
8	Resistant	Resistant	Susceptible	Susceptible

In the present study, higher sensitivity (100%) for detection RIF and INH resistance were observed indicating that most of the mutations conferring RIF and INH resistance were found in the region that were incorporated in the LPA strip. However, the specificity of LPA in our study for detecting RIF-resistant was lower than INH resistance and other previous studies[13,17,19,21,22].

Variability in the present assay can be explained by regional differences in RIF and INH resistance mutation frequencies[23]. LPA identified 7 RIF and 5 INH resistance from 8 samples, but no resistant strain

Table 3. Mutation detected by GenoType MTBDR*plus* assay compared with phenotypic culture results from 13 specimens

		Gene region/specific mutation			Conventional DST	
No.	Band missing Mutation band	***rpo*B**	***Kat*G**	***inh*A**	RIF	INH
1	WT8	530-533			S	
	MUT1			C15T		S
2	WT8	530-533			S	S
3	WT8	530-533			S	
	MUT1			C15T	S	S
4	MUT1			C15T	S	S
5	WT8	530-533			R	S
	MUT3	S 531L			R	S
6	WT8	530-533			S	S
7	*kat*GWT		315		S	R
	MUT1		S315T1		S	R
8	WT8	530-533			S	S
9	WT3, WT4	513-519			R	S
10	*kat*GWT		315		S	R
	MUT1		S315T1		S	R
11	WT8	530-533			S	S
12	WT8	530-533			S	
	MUT1			C15T		S
13	*kat*GWT		315		S	R
	MUT1		S315T1		S	R

8 out of 13 RIF-resistant strain showed loss of wild type band 8

LPA = Line probe assay, No = Number, S = Sensitive, R = Resistant

WT8 = Wild type band 8

WT3/4 = Wild type band 3 and 4

detected from conventional method. Discordance between LPA and conventional method because the LPA only screens the nucleic acid sequence and not the amino acid sequence, so it is possible that mutation in the probe region that do not cause an amino acid exchange or silent mutation will still absence of one of the the wild type bands without resistance by conventional method. This is the limitation of LPA includes their inability to differentiate between resistance inducing and silent mutation that leads to false positive resistance interpretation. In the present study, the missing wild type probe 8 [WT8] without any mutant bands was found in 7 from 13 (53.8%) of RIF-resistant isolates, which was reported more than other studies such as in India (26.1%)[24], France (29%)[16] and Vietnam (33.3%)[25]. It was the the most common mutation in RIF-resistant strains which still be RIF-sensitive by conventional method in 87.5%. Therefore, if the band WT8 was missing and the *rpo*B mutation band did not develop, conventional culture and DST should be considered.

Moreover, in case of mixed susceptible and resistant population infection (presence of all wild type bands along with presence of one or more mutant bands) may cause discordant between LPA and conventional method. There was only one specimen of mixed infection (Infection with 2 different strains, a wild-type and drug-resistant strain) of INH resistance in the present study. (Specimen number 4 in Table 3: All wild type bands are present and one of the mutant bands is also present). In the present study, the LPA detected the INH-mutant band without detecting INH resistance by conventional DST.

Among INH-resistant strains, both *inh*A and *kat*G mutations were seen in 4/54 (7.4%) and 3/54 (5.5%) of total specimens (Table 3). However, mutations at *inh*A gene failed to present resistance by conventional DST. All of the INH-resistant strains were detected at *kat*G mutations that account for commonest mechanism of INH resistance, while mutation in *inh*A gene is considered for low level INH resistance. No missing wild type band in INH-resistant strains found in this study.

The limitation of the present study is that it involved a relatively small number of specimens which may have an insufficient drug-resistant samples to detect the differences of performances between LPA and conventional DST for detecting INH resistance, RIF resistance and MDR-TB.

Conclusion

In conclusion, the GenoType MTBDR*plus* assay has been a highly sensitive and quite specific diagnostic test for detecting RIF and especially INH resistance. There are some limitations of LPA in case of silent mutations or mutations not conferring resistance that LPA lead to false resistance report.

What is already known on this topic?

LPA was recommended by the WHO for the diagnosis of MDR-TB in 2008. Meta-analyses have shown that LPA are highly accurate for the detection of drug resistance, especially in smear positive specimens. They also showed that LPA are highly sensitive (≥97%) and specificity (≥99%) for the detection of RIF mono-resistance or in combination with INH resistance (Sensitivity ≥90% and specificity ≥99%) on isolates and on smear-positive sputum specimens. One study that studied in Thailand showed that GenoType MTBDR*plus* assay had a sensitivity of 95.3%, 100% and 94.4% for detection of INH resistance, RIF resistance and MDR-TB, respectively. Specificity was 100% for all resistant patterns.

What this study adds?

In the present study showed variability of LPA in different settings across the world. The LPA from this study was shown to have excellent sensitivity (100%) for both RIF and INH resistance but specificity of the assay was lower than previous studies, (86.5% for RIF resistance and 92.1% for INH resistance). It showed that not all mutations are associated with phenotypic resistance. Mutation in the probe region that do not cause an amino acid exchange or silent mutation, therefore no resistance was detected by conventional culture. Moreover, in case of mixed susceptible and resistant population infection may cause discordant between LPA and conventional method.

Potential conflicts of interest

The authors declare no conflict of interest.

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