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ORIGINAL ARTICLE

The reliability of a rapid molecular detection method in determining the prevalence of rifampicin-resistant *Mycobacterium tuberculosis* in an urban district health facility in Malaysia

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Abstract

Introduction: Rifampicin is a key first-line antimycobacterial agent employed for the treatment of pulmonary tuberculosis (PTB). This study sought to obtain prevalence data on rifampicin-resistant *Mycobacterium tuberculosis* among smear-positive PTB patients in the Klang District of Malaysia. *Materials and Methods*: A total of 103 patients from the Chest Clinic of Hospital Tengku Ampuan Rahimah with sputum smears positive for acid-fast bacilli were included in this cross-sectional study. All sputa were tested using Xpert MTB/RIF to confirm the presence of *M. tuberculosis* complex and detect rifampicin resistance. Sputa were also sent to a respiratory medicine institute for mycobacterial culture. Positive cultures were then submitted to a reference laboratory, where isolates identified as *M. tuberculosis* complex underwent drug susceptibility testing (DST). *Results*: A total of 58 (56.3%) patients were newly diagnosed and 45 (43.7%) patients were previously treated. Xpert MTB/RIF was able to detect rifampicin resistance with a sensitivity and specificity of 87.5% and 98.9%, respectively. Assuming that a single resistant result from Xpert MTB/RIF or any DST method was sufficient to denote resistance, a total of 8/103 patients had rifampicin-resistant *M. tuberculosis.* All eight patients were previously treated for PTB ($p<0.05$). The overall prevalence of rifampicin resistance among smear-positive PTB patients was 7.8%, although it was 17.8% among the previously treated ones. *Conclusion*: The local prevalence of rifampicin-resistant *M. tuberculosis* was particularly high among previously treated patients. Xpert MTB/RIF can be employed in urban district health facilities not only to diagnose PTB in smear-positive patients, but also to detect rifampicin resistance with good sensitivity and specificity.

Keywords: Mycobacterium tuberculosis, pulmonary tuberculosis, rifampicin, Xpert MTB/RIF

INTRODUCTION

Worldwide, *Mycobacterium tuberculosis* is the leading cause of mortality attributable to a single infectious agent (after HIV) and resulted in more than a million deaths in 2017.[1] *M. tuberculosis* isolates with rifampicin resistance are regarded as multidrug-resistant (MDR) strains which threaten global tuberculosis control efforts because >90% of these isolates are also isoniazid-resistant.[2] Approximately 3.5% of new tuberculosis cases and 18% of previously treated cases are caused by MDR strains.[1] The conventional and gold standard approach to laboratory detection of drug resistance in *M. tuberculosis* is essentially phenotypic, via media-based DST methods which often take weeks before results are available.[3] Although highly specific, phenotypic assays suffer from low sensitivity because they depend on the presence of viable organisms.

Thus, a rapid and sensitive method of detecting drug resistance is desirable to ensure that individuals with MDR strains are correctly diagnosed and started on the

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appropriate (e.g. second-line) drugs as soon as possible to prevent the dissemination of the infection within the community. There is a fully automated commercial cartridge-based nucleic acid amplification test kit which can detect both *M. tuberculosis* complex and rifampicin resistance known as Xpert MTB/RIF (Cepheid, Sunnyvale, USA) within two hours. This kit is widely available in tertiary and state hospitals in Malaysia, although not as a point-of-care test due to the need to process sputa in a biosafety cabinet beforehand. The kit has been previously evaluated for use in district and subdistrict health facilities in low-resource settings, with satisfactory results.[4] The sensitivity of Xpert MTB/RIF in diagnosing PTB is affected by smear positivity, with reported sensitivity values of 77% and 99% in smear-negative and smear-positive patients, respectively.[4,5] The objectives of this study were to evaluate the performance of Xpert MTB/RIF and obtain prevalence data on rifampicin-resistant *M. tuberculosis* among smear-positive PTB patients in the Klang District of Malaysia.

MATERIALS AND METHODS

Study design and participants

This prospective cross-sectional study was conducted over a period of two years, from April 2017 till April 2019 in the Chest Clinic as well as the Medical Microbiology Laboratory of Hospital Tengku Ampuan Rahimah (HTAR) in the Klang district of Malaysia. Patients above the age of 18 years who had at least one smear-positive sputum specimen were randomly enrolled in the study. The participation of all study subjects was voluntary, and informed consent was obtained from each participant. The study was approved by the Research and Ethics Committee of Universiti Kebangsaan Malaysia (approval code: FF-2017-226). Ethical approval was also obtained from the Medical Research and Ethics Committee (MREC), Ministry of Health, Malaysia. Data on patient demographics and past medical history (in particular, HIV status and previous history of PTB treatment) were collected from each patient. Patients who were previously treated for PTB were also asked if they managed to complete their treatment courses.

Testing protocols

Each sputum sample collected from the chest clinic of HTAR was stained with Auramine O and examined using a fluorescence microscope. Only sputum samples which were smear-positive (i.e. displaying acid-fast bacilli of at least 1+) were subjected to the Xpert MTB/RIF assay, as per the manufacturer's instructions. This fully automated sample processing and real-time PCR assay amplifies an *rpoB* gene sequence which is specific to *M. tuberculosis* complex members, as well as probes for mutations within the 81-bp rifampicin resistance-determining region of the *rpoB* gene. A sample was considered *M. tuberculosis*-positive if at least two of the five *rpoB* probes were positive within two cycles of each other. Resistance to rifampicin was deduced from the inability of at least one of the *rpoB*-specific molecular beacons to hybridize correctly to the *rpoB* amplicon.

At the same time, sputum samples from the Chest Clinic of HTAR were also sent to the Respiratory Medicine Institute (IPR) in Kuala Lumpur for mycobacterial culture in either liquid media (i.e. Mycobacteria Growth Incubator Tube or MGIT) or solid media (i.e. Löwenstein-Jensen or LJ agar), regardless of smear positivity or the Xpert MTB/RIF assay result. All positive cultures from IPR were then sent to the National Public Health Reference Laboratory (MKAK) in Sungai Buloh, Malaysia for isolate identification and DST. Like IPR, the reference laboratory was also not privy to the isolates' Xpert MTB/RIF results.

In MKAK, all positive cultures were subjected to the Capilia TB-Neo immuno-chromatographic assay (TAUNS Laboratories, Japan) to identify and differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria. However, if an isolate could not be identified, the AccuProbe *Mycobacterium tuberculosis* complex nucleic acid hybridisation assay (Gen-Probe Incorporated, USA) was used as a second-line identification assay.

All *M. tuberculosis* complex-confirmed isolates were then subjected to DST using either the absolute concentration method on LJ agar or the broth-based proportion method using BACTEC MGIT 960 SIRE kits (Becton Dickinson Biosciences, USA). The choice of broth- or agar-based DST depended on whether the specimen was submitted to MKAK on LJ agar or in MGIT media. For specimens that were already grown on LJ agar, those with good colonial growth were subjected to broth-based DST and those with poorer growth were tested using the agar-based method. However, for specimens submitted in MGIT media, broth-based DST was chosen, although each specimen was also subcultured on LJ agar as backup.

Occasionally, the chest clinic physicians sent sputum specimens directly to MKAK for DST. Usually, such specimens belonged to patients who were already known to have pulmonary tuberculosis and thus the chief concern was to detect drug resistance rather than to diagnose the infection. Since these specimens bypassed IPR, they were not cultured. In MKAK, these specimens were subjected to the GenoType MTBDRplus VER 2.0 (Hain Lifescience GmbH, Germany) DNA•STRIP line probe assay which had the dual capability of identifying members of *M. tuberculosis* complex as well as detecting drug resistance.

Interpretation of drug resistance

With the notable exception of the Xpert MTB/RIF assay which could only test for rifampicin resistance, all the other three DST methods done in MKAK were also able to at least test for isoniazid resistance. With the LJ agar-based absolute concentration method, resistance was defined as growth of 1+ or more (≥20 colonies) in the drug-containing agar when compared to the control (drug-free) agar. Each drug-containing agar was dosed with the critical concentration of an antimycobacterial agent, i.e. 40 μg/mL for rifampicin and 0.2 μg/mL for isoniazid.

Using the broth-based proportion method, resistance was detected when the growth unit value (determined by the BACTEC MGIT 960 system based on the principle of increased fluorescence as a result of oxygen depletion due to active mycobacterial growth) of the drug-free control tube had reached at least 400 and the growth unit value of the drug-containing tube was at least 100. For the broth-based method, the critical drug concentrations used were 1 μg/mL for rifampicin and 0.1 μg/mL for isoniazid.

Lastly, the DNA•STRIP assay detected drug resistance in an isolate when at least one of various "wild type" probes (8 for rifampicin and 3 for isoniazid) were unable to bind to their amplicons. This interpretation was based on the logic that "wild type" drug susceptibility gene(s) must not be mutated for the isolate to remain susceptible to the corresponding drug.

RESULTS

Sociodemographic data of study subjects

A total of 103 patients who fulfilled the inclusion criteria were included in this 2-year study. All had sputa which were also positive for *M. tuberculosis* complex by Xpert MTB/RIF. Out of these 103 patients, 58 (56.3%) were newly diagnosed PTB patients and 45 (43.7%) were patients who had previously received treatment for PTB. Table 1 shows the socio-demographics

TABLE 1: Sociodemographic data of study subjects

	Total (n=103)	Newly diagnosed (n=58)	Previously treated (n=45)
Gender			
Male	73 (70.9%)	40 (69.0%)	33 (73.3%)
Female	30 (29.1%)	18 (31.0%)	12 (26.7%)
Age groups (years)			
<20	4 (3.9%)	2 (3.5%)	2 (4.4%)
20-29	20 (19.4%)	12 (20.7%)	8 (17.8%)
30-39	27 (26.2%)	17 (29.3%)	10 (22.2%)
40-49	20 (19.4%)	9 (15.5%)	11(24.4%)
50-59	18 (17.5%)	8 (13.8%)	10 (22.2%)
>60	14 (13.6%)	10 (17.2%)	4 (8.9%)
Ethnicity			
Malay	48 (46.6%)	27 (46.6%)	21 (46.7%)
Chinese	19 (18.4%)	10 (17.2%)	9 (20.0%)
Indian	18 (17.5%)	8 (13.8%)	10 (22.2%)
Foreigner	18 (17.5%)	13 (22.4%)	5 (11.1%)
HIV Status			
Positive	6 (5.8%)	2 (3.4%)	4 (8.9%)
Negative	97 (94.2%)	56 (96.6%)	41 (91.1%)

of the patients with regards to gender, age, ethnicity and HIV status. For both groups of patients, males predominated, most were of Malay ethnicity and patients were mostly HIV-negative. The median age of newly diagnosed patients was only marginally lower (38 vs. 40 years) compared to previously treated patients.

Detection of rifampicin resistance

Out of 103 specimens assayed with Xpert MTB/RIF, seven were found to contain rifampicin-resistant *M. tuberculosis* strains. One specimen had rifampicin-susceptible mycobacteria based on the Xpert MTB/RIF result but the bacteria were found to be resistant to rifampicin when tested using the agar-based absolute concentration method. Out of the seven specimens containing mycobacteria labelled as rifampicin-resistant by Xpert MTB/RIF, one was found to actually contain rifampicin-susceptible mycobacteria when tested using alternative DST methods (i.e. the broth-based proportion method and the DNA•STRIP assay). As presented in Table 3, using any of the three alternative DST methods (i.e. the agar-based absolute concentration method, the broth-based proportion method or the DNA•STRIP assay) as the reference method, Xpert MTB/RIF was able to detect true rifampicin resistance with a sensitivity of 87.5% and a specificity of 98.9%.

However, with the assumption that a single resistant result obtained through any of the four DST methods (i.e. Xpert MTB/RIF assay, agar-based absolute concentration method, broth-based proportion method or DNA•STRIP assay) was already sufficient to denote resistance, a total of eight patients had sputa containing rifampicin-resistant *M. tuberculosis*. All eight specimens belonged to the "previously treated" patient group ($p<0.05$). Four patients completed their treatment courses while the remaining four were deemed to be treatment defaulters. Table 2 shows the characteristics of patients with rifampicin-sensitive and rifampicin-resistant *M. tuberculosis* strains. Thus, the overall prevalence of rifampicin resistance amongst *M. tuberculosis* isolates from smear-positive PTB patients in our local setting was 7.8%. While the prevalence of

TABLE 2: Patient characteristics based on the susceptibility of M. tuberculosis to rifampicin

	Rifampicin-resistant (n=8)	Rifampicin-sensitive (n=95)	*p*-value
Mean age (years) ± SD	34 ± 12	42 ± 15	0.145[a]
Age groups (years)			
<30	3 (37.5%)	21 (22.1%)	0.385[b]
≥30	5 (62.5%)	74 (77.9%)	
Previously treated			
Yes	8	37	0.001[b]
No	0	58	
Gender			
Male	5	68	0.689[b]
Female	3	27	
Ethnicity			
Malay	4	44	0.910[b]
Chinese	1	18	
Indian	1	17	
Foreigner	2	16	
HIV Status			
Positive	1	5	0.392[b]
Negative	7	90	

[a] Derived from 2-tailed unpaired t-test

[b] Derived from Fischer's exact test

TABLE 3: Sensitivity and specificity of Xpert MTB/RIF in detecting rifampicin resistance

		Reference DST method	
		Rifampicin resistant	**Not rifampicin resistant**
Xpert MTB/RIF	Rifampicin resistance detected	6	1
	Rifampicin resistance not detected	1	95
	Sensitivity	87.5%	
	Specificity	98.9%	

rifampicin-resistant *M. tuberculosis* amongst newly diagnosed smear-positive PTB patients was nil, its prevalence among those who were previously treated was much higher at 17.8%.

Reliability of Xpert MTB/RIF in predicting MDR-TB

When the seven specimens with mycobacteria labelled as rifampicin-resistant by Xpert MTB/RIF had their isolates' rifampicin susceptibility tested by alternative methods, it was found that only five specimens contained strains which were resistant to both rifampicin and isoniazid, while the remaining two specimens had strains which were either rifampicin monoresistant (1 specimen) or were sensitive to both drugs (1 specimen). As presented in Table 4, using rifampicin resistance as a surrogate marker or proxy for multidrug resistance, Xpert MTB/RIF was able to detect isolates which were truly MDR strains (i.e. also confirmed to possess isoniazid resistance) with a sensitivity of 100%, although its specificity was much lower at 33.3%.

DISCUSSION

Rifampicin is an effective antimycobacterial agent against both actively metabolizing and slowly metabolizing bacilli. It binds to the β subunit of the RNA polymerase enzyme (encoded for by the *rpoB* gene) to inhibit mRNA elongation in mycobacteria.[3] Rifampicin resistance in *M. tuberculosis* is a natural phenomenon, as a consequence of spontaneous and random mutations typically occurring at a rate of 2.25×10^{-10} per bacterium per generation.[6] Thus, the *M. tuberculosis* population within a given human host is likely to harbour a small proportion of naturally occurring drug-resistant mutant strains which will be selected for under conditions of inadequate (or ineffective) drug therapy. Compared to other first-line agents (i.e. ethambutol, isoniazid and streptomycin), the mutation rate for rifampicin appears to be the lowest, with the highest rate being recorded for ethambutol, followed by isoniazid and streptomycin.[6] This is probably why resistance to rifampicin is apt as a surrogate marker for multidrug resistance, because by the time a bacterium is rifampicin-resistant, it would have already accumulated the necessary mutations which would also render it resistant to other drugs. Echoing this is the observation that

TABLE 4: Sensitivity and specificity of Xpert MTB/RIF in detecting MDR mycobacterial strains

		Reference DST method	
		Resistant to both rifampicin and isoniazid	**Resistant to rifampicin, but susceptible to isoniazid**
Xpert MTB/RIF	Rifampicin resistance detected (proxy for multidrug resistance)	5	2
	Rifampicin resistance not detected	0	1
	Sensitivity	100.0%	
	Specificity	33.3%	

isoniazid mono-resistance is a lot more common than rifampicin mono-resistance.[7]

Although the district of Klang has its share of rifampicin-resistant *M. tuberculosis* strains, such strains were only detected in patients who had received prior treatment for PTB. It would seem logical that drug-resistant strains would only emerge in PTB patients who were poorly compliant to their past treatment regimens as a consequence of drug selection pressures. However, in our setting, a history of completing a previous treatment regimen did not appear to significantly impede the development of resistant strains because half of our patients with such strains actually successfully completed their treatment regimens. This suggests that even amongst compliant patients, other factors also play significant roles in achieving complete treatment success. A likely contributing factor for treatment failure in such patients is the fact that different individuals metabolize antimycobacterial drugs at different rates when the drugs are administered at standard doses. Patients classified as rapid acetylators are at higher risk of treatment failure regardless of the number of antimycobacterial agents prescribed in the treatment regimen.[8]

Despite proposals to consider rifampicin resistance as a surrogate marker for multi-drug resistance, local antimycobacterial DST data may not necessarily be in absolute support of this. Globally, rates of rifampicin mono-resistance vary greatly, from as high as 10% in South Africa to as low as 0.12% in France.[7,9] From our own data, three out of 103 *M. tuberculosis* isolates had rifampicin mono-resistance, resulting in a rate of 2.9%. However, once an isolate is found to be resistant to rifampicin (regardless of its resistance to additional drugs), the patient should be considered to have multidrug-resistant tuberculosis (MDR-TB) and a protracted treatment course of at least 20 months should be contemplated.[10] Thus, from the practical point of view, the Xpert MTB/RIF kit should be able to assist respiratory physicians in offering the most suitable treatment regimen to patients with PTB by only taking an isolate's resistance to rifampicin into account. The performance of Xpert MTB/RIF in detecting rifampicin resistance in our setting appears to be quite similar to that reported by Cape Town investigators; with reported sensitivity and specificity values of 90% and 98%, respectively.[4] This essentially means that although the Xpert MTB/RIF kit is suitable as a screening tool, it is particularly apt as a confirmatory test for rifampicin resistance.

There are several risk factors thought to be associated with MDR-TB. Since rifampicin susceptibility can be regarded as a game changer when planning PTB treatment regimens, for the purpose of discussion, "rifampicin-resistant" in Table 2 will also be considered as "MDR". While gender has been reported to be a risk factor for MDR-TB, the exact gender varies depending on the study location. For instance, males appear to be more at risk in Israel and females have a higher risk for MDR-TB in Karachi.[11,12] Our own study did not show any statistically significant association between gender and drug resistance. Several studies have reported that younger patients are at higher risk of MDR-TB, possibly due to poorer treatment compliance in these patients.[13] However, the precise definition of "young" is inconsistent and appears to be study-dependent. A Chinese study labelled individuals belonging to the age group of 35-44 years as "young" while an Ethiopian study classified "young" as being below 30 years.[13,14] However, our study showed no significant association between young age and drug resistance, although the mean age of patients with drug-resistant mycobacteria was lower. A Pakistani study reported that certain ethnic groups are at higher risk of MDR-TB, namely the Sindhis and Pashtoons.[11] Thus, we also attempted to ascertain if drug resistance was more prevalent in any of the major ethnic groups in Malaysia but found no significant association. Being co-infected with HIV has been identified as a predictor or risk factor for MDR-TB by some studies,[12,13] although this was not the case with our patients. Finally, only a previous treatment history was found to be a statistically significant predisposing factor for drug resistance in our patients, and is likely to be due to reasons which have already been discussed earlier. This positive finding of ours is consistent with that of numerous other studies from across the globe.[11-14]

CONCLUSION

The prevalence of rifampicin resistance among *M. tuberculosis* isolates from smear-positive PTB patients in the Klang district of Malaysia is relatively high, with rates approaching 8% and 18% among all cases and previously treated cases, respectively. Due to the poor reliability of clinical and demographic predictors of

rifampicin resistance (with the exception of past treatment history), the capability to objectively detect rifampicin resistance using an established laboratory method/test is highly desirable. The Xpert MTB/RIF is a commercially available molecular-based diagnostic kit which can be utilised in urban district health facilities not only to reliably diagnose PTB in smear-positive patients, but also detect rifampicin resistance with good sensitivity and excellent specificity, as well as MDR-TB with excellent sensitivity. The fact that Xpert MTB/RIF is a rapid test may also theoretically aid infection control efforts by allowing the appropriate treatment regimen to be instituted early (e.g. during the same clinic visit).

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