Detection of *Mycobacterium tuberculosis* and drug resistance by GeneXpert® MTB/RIF and Anyplex™ II MTB/MDR in a tertiary care hospital

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ABSTRACT

Tuberculosis (TB) is a major global health threat, aggravated by emergence of drug resistance. Early diagnosis is critical and has been offered lately by molecular assays. This study aimed to evaluate the performance of GeneXpert® MTB/RIF and Anyplex™ II MTB/MDR assays in simultaneous direct detection of *Mycobacterium tuberculosis* (MTB) and genetic determination of Rifampicin (RIF) resistance. This study was conducted with 100 specimens collected from TB suspects. ZN (Zeil-Neelsen) smears were prepared and L-J (Lowenstein-Jensen) culture was used for isolation of MTB. GeneXpert and Anyplex assays were performed directly on specimens to detect *Mycobacterium tuberculosis* (MTB) and RIF resistance. MTB was isolated on L-J culture from 40% of specimens. For MTB detection, Xpert and Anyplex assays showed same total sensitivity of 95%, and specificities of 73.3% and 81.7%, respectively. Rifampicin resistance was detected by Xpert and Anyplex assays with rates of 5.5% and 6.12%, respectively showing significant agreement with a P-value < 0.001. Time of assay was 2 hours for Xpert and 3 hours for Anyplex. In conclusion, both molecular assays enable rapid concomitant detection of MTB and drug resistance with high sensitivity, specificity and significant agreement. Xpert showed more technical ease, however higher cost than PCR.

Key words: MTB; Molecular; Resistance; PCR; Rifampicin; Xpert

1. INTRODUCTION

Infection with *Mycobacterium tuberculosis* (MTB) is considered a major threat to public health and is associated with high rates of morbidity and mortality. Moreover, the emergence of drug-resistant strains has increased concern, with their infection resulting in treatment failure and poor clinical outcomes (Sali et al., 2016). In 2017, tuberculosis (TB) was listed among the 10 most common causes of death worldwide; there were an estimated 10 million new cases, with 1.6 million resulting in death. Globally, rifampicin resistance developed in 558,000 cases in 2017, and of these, 82% were proven to be multidrug-resistant TB (MDR-TB), (WHO, 2018). Rapid diagnosis of TB enables prompt patient management and reduces disease transmission; thus, it is considered crucial for effective disease control. TB culture using Lowenstein Jensen (L-J) medium is considered the gold standard method for MTB detection and susceptibility testing for anti-TB drugs added to L-J media in the agar proportion method. Other conventional methods, such as the mycobacterial growth inhibition assay (MGIA) (manual or automated), have been developed and exhibit high sensitivity and specificity in MTB detection and drug susceptibility testing. However, these conventional methods are limited by a long time required for detection with additional time needed for drug susceptibility testing after obtaining a primary isolate in culture. These characteristics prevent rapid delivery of results and early management (Pandey et al., 2016). Moreover, these methods are laborious and require well-trained staff. Nonetheless, early diagnosis of TB has been greatly facilitated by the evolution of molecular methods that allow direct simultaneous MTB detection and susceptibility testing using clinical samples (Caulfield and Wengenack, 2016). One of these molecular methods is the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), which was endorsed and recommended by the WHO for rapid diagnosis of TB in 2010. GeneXpert is capable of simultaneously providing information about direct MTB detection and susceptibility to rifampin (RF) in 2 hours using clinical samples (Albert, 2016).

Another rapid diagnostic method for multidrug-resistant TB (MDR-TB) is the Anyplex MTB MDR assay, which is a multiplex real-time PCR method that allows concomitant detection of MTB and its genetic determinants of resistance to RF and isoniazid (INH) (Sali et al., 2016).

This study aimed to evaluate Anyplex™ II MTB/MDR and GeneXpert molecular assays in MTB detection compared to the gold standard L-J culture method and to determine the agreement of both molecular assays in testing susceptibility of MTB to RF.

2. METHODOLOGY

Specimen collection

The present study was conducted using one hundred specimens of different types collected from patients who were highly suspected or known to have pulmonary or extra-pulmonary TB in a tertiary chest hospital in the period from October 2017 to July 2018 (Figure 1). All specimens were collected and transported to the mycobacteriology laboratory in sterile, leak-proof containers with adherence to required standard biosafety measures. This study was approved by the Research Ethics Committee of the Clinical and Chemical Pathology Department, Cairo University.
Smear examination
Acid-fast bacillus microscopy using ZN stain was performed on all specimens and interpreted according to Yadav et al., 2016. Smears were prepared from the original sample before processing (from the thick purulent part of the specimen) (Yadav et al., 2016).

Specimen processing
Non-sterile specimens were decontaminated using the standard N acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method (a solution of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate was used), which is consistent with all culture media and PCR techniques (Asmar and Drancourt., 2015). The NALC/NaOH solution was added to the specimen in screw-capped plastic centrifuge tubes, and the mixtures were centrifuged at 3000 x g for 15 minutes. All steps were repeated 3 times. The supernatant was discarded, and then the concentrated sediment was suspended in 1.5 ml sterile phosphate buffer (pH 7.0) (Alfred et al, 2014) to be used for culture, Anyplex PCR and GeneXpert MTB/RIF assays.

Culture on L-J medium
All specimens were cultured on solid L-J medium for MTB isolation. Bottles of L-J medium were manually prepared and inoculated with 0.1 ml of the resuspended specimen and then incubated at 37°C. Routine inspection for mycobacterial growth was performed at least once a week for 8 weeks or until mycobacterial colonies were detected. MTB was identified according to ZN-smear microscopy and the colony morphology growth rate, in addition to standard biochemical tests, nisin production and nitrate reduction. Non-tuberculous Mycobacterium (NTM) was excluded by the use of the para-nitrobenzoic acid (PNB) inhibition test, in which MTB is susceptible, resulting in growth inhibition, but NTB resistant. Sterility and performance checks of L-J bottles were performed using a quality control MTB strain (H37RV).

GeneXpert® MTB/RIF
All specimens were tested using the GeneXpert MTB/RIF assay for simultaneous detection of MTB and its resistance to RF through detection of a segment of 192 bp corresponding to a known region of the rpoB gene containing the localized RF resistance region in an 81-bp segment called RRDR. The procedure was performed according to the manufacturer’s instructions (Cepheid, Sunnyvale, USA). An internal control (lyophilized Bacillus globigii) and five molecular beacon hybridization probes corresponding to the target sequences of the rpoB gene for the same multiplex reaction are provided by the manufacturer.

Anyplex™ II MTB/MDR Detection assay
The Anyplex™ II MTB/MDR Detection assay (Seegene Incorporated, Korea) was used for the simultaneous detection of MTB and its resistance to first-line anti-tuberculosis drugs (INH and RIF). The assay covers 7 mutations in the katG gene and inhA promoter region causing INH resistance and 18 mutations in the rpoB gene causing RF resistance. By applying Seegene’s DPO™ and TOCE™ technologies without additional downstream processing, each drug-resistant target mutation is amplified and detected specifically by corresponding oligonucleotides within 3 hours. PCR was performed using CFX96 Real-time PCR System (Bio-Rad) according to the manufacturer’s instructions. Negative (RNase-free water), positive (mixture of clones of all positive targets and internal control) and wild-type (mixture of clones of wild-type MTB targets and internal control) controls were included in each test run. All results were interpreted according to the cutoff values determined by the manufacturer (Sali et al., 2016).

Data analysis
Statistically, data were described as mean ± standard deviation (± SD), and range, or frequencies (number of cases) and percentages when appropriate. Comparison between the study groups was made using Chi-square (X²) test. P values less than 0.05 was considered statistically significant. SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows was used for statistical calculations.

3. RESULTS
The hundred specimens used in this study were distributed as shown in (Table 1), with the highest proportions for respiratory samples (95/100), which occurred in the form of sputum, broncho-alveolar and pleural fluid with frequencies of 70, 22 and 3, respectively. History of antituberculous treatment was recorded in 52% of patients included in our study.
Table (1): Types of specimens tested.

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>Frequency (n)</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>BAL</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CSF</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pus from abscess</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Urine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

3.1. MTB detection

MTB was isolated from 40 of the 100 specimens using the gold standard L-J medium. Smears were positive for acid-fast bacilli in 30% of samples. Of these, 23 were smear positive and culture positive, 17 were smear negative and culture positive, and 7 were smear positive and culture negative, showing sensitivity and specificity of 57.5% and 88.3%, respectively.

GeneXpert and Anyplex PCR showed positive MTB detection in 54 and 49 specimens, respectively. Compared to the results of the gold standard L-J method, the sensitivity and specificity were 95% and 73.3% for GeneXpert, respectively, and 95% and 81.7% for Anyplex PCR, respectively, as described in (Table 2 and Figure 2). The sensitivities of GeneXpert and PCR were 100% and 96.1%, respectively, for smear-positive specimens, and 88.2% and 94.1%, respectively, for smear-negative specimens. The GeneXpert and Anyplex PCR positive and negative results agreed in 46 and 43 specimens, respectively, which was significant (P-value <0.001). Discrepant results were observed in 11 samples, 8 (GeneXpert+/PCR-) and 3 (GeneXpert-/PCR+).

Table (2): Results of MTB detection by GeneXpert and Anyplex PCR compared to the gold standard L-J culture method.

<table>
<thead>
<tr>
<th>Results</th>
<th>GeneXpert versus L-J method</th>
<th>Anyplex PCR versus L-J method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Number</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>Specificity</td>
<td>73.3%</td>
<td>81.7%</td>
</tr>
<tr>
<td>NPV</td>
<td>70.4%</td>
<td>77.6%</td>
</tr>
<tr>
<td>PPV</td>
<td>95.7%</td>
<td>96.1%</td>
</tr>
</tbody>
</table>

NPV: Negative predictive value, PPV: Positive predictive value

3.2. Susceptibility to RIF and INH

Table 3 summarizes the results of MTB susceptibility to RIF by GeneXpert and to RIF and INH by Anyplex PCR. Resistance to RIF was detected in 3 of the 54 MTB-positive samples detected by GeneXpert, accounting for 5.5%. In contrast, 3 of the 49 MTB-positive samples detected by Anyplex PCR were resistant to RIF and INH, accounting for 6.12%. Isoniazid resistance was detected in 9 out of 49 (18.36%) MTB positive samples by Anyplex PCR; where 6 samples showed INH mono-resistance (12.2%), while 3 showed resistance to both INH/RIF (Figure 3). The total 9 INH resistant MTB samples were divided into 5 and 4 that were mediated by katG and inhA promoter mutations, respectively. Among the 46 MTB-positive cases commonly detected by GeneXpert and Anyplex PCR, 44 specimens showed agreement with regard to the results of RIF susceptibility, with significance (P-value <0.001); however, 2 cases showed discrepant results (Table 3).

Table (3): The Susceptibility profile of MTB to RIF by GeneXpert and to RIF and INH by Anyplex PCR.

<table>
<thead>
<tr>
<th>Number of specimens</th>
<th>GeneXpert MTB +ve (n= 54)</th>
<th>Anyplex PCR MTB +ve (n= 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R I</td>
<td>R I</td>
</tr>
<tr>
<td>2</td>
<td>R S</td>
<td>R S</td>
</tr>
<tr>
<td>36</td>
<td>S S</td>
<td>S S</td>
</tr>
<tr>
<td>6</td>
<td>S S</td>
<td>S R</td>
</tr>
<tr>
<td>1</td>
<td>R S</td>
<td>R S</td>
</tr>
</tbody>
</table>
The summary of the evaluation of GeneXpert and Anyplex PCR for MTB detection compared to the gold standard L-J method test with regard to sensitivity, specificity, labour, cost and time to detection is described in (Table 4).

Table (4): Evaluating GeneXpert and Anyplex PCR for MTB detection compared to the gold standard L-J method.

<table>
<thead>
<tr>
<th>Test method</th>
<th>Labour</th>
<th>Cost/ test ($)</th>
<th>Time to detection</th>
<th>Sensitivity/Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJ</td>
<td>Moderate</td>
<td>2.96</td>
<td>6-8 weeks</td>
<td>-</td>
</tr>
<tr>
<td>GeneXpert</td>
<td>Easy</td>
<td>42.4</td>
<td>2 hours</td>
<td>95/73.6</td>
</tr>
<tr>
<td>Anyplex II</td>
<td>Moderate</td>
<td>24.2</td>
<td>3 hours</td>
<td>95/81.7</td>
</tr>
<tr>
<td>MTB/MDR PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MTB: *Mycobacterium tuberculosis*; RIF: rifampin; INH: isoniazid; R: resistance; S: sensitive; \(^a\): MTB negative by GeneXpert; \(^b\): MTB negative by Anyplex PCR; \(*\) significant P-value is less than 0.005

Figure 1: Flow chart for the specimen laboratory workup

Figure 2: Compared analytical sensitivity and specificity of Xpert MTB/RIF and AnyplexII MTB/MDR assays
4. DISCUSSION

TB is a communicable disease that is spreading worldwide with high yearly estimates of infection and high fatality rates (WHO, 2018). In the present study, MTB was recovered in nearly half of the specimens evaluated, with a rate of 40% by the gold standard L-J culture method. The high rate of MTB recovered in the present study reflects the high burden of MTB infection in low-income countries, as more than 90% of new TB cases and deaths occur in developing countries (WHO, 2018). In Egypt, the incidence rate of TB was 13 per 100,000 people in 2017, as estimated by the World Health Organization (WHO, 2018). However, in the present study, the inclusion of specimens from patients already clinically and radiologically diagnosed as having TB might have contributed to the high recovery rate. In contrast, the TB burden is low in high-income countries with high socioeconomic standards due to high-resource settings that offer adequate diagnosis and treatment and apply optimum measures for disease control (Chugh, 2017).

In the present study, the vast majority of the specimens were of respiratory origin, accounting for 95%, in line with the usual higher proportion of pulmonary to extra pulmonary TB related to the nature of the airborne transmission of the disease (Lee, 2016). We used the ZN smear assay, L-J gold standard culture method, GeneXpert and Anyplex PCR to detect MTB. Although ZN smear is a rapid and inexpensive method, it is limited by low sensitivity due to the need for a high bacillary load (5000-10,000 bacilli/mm of specimen) and low specificity due to the inability to differentiate between viable and dead bacilli in patients who are under treatment or between MTB and NTM (Hooja et al., 2011; Kang et al., 2016). This situation was emphasized by our results, where ZN smear showed a sensitivity and specificity of 57.5% and 88.3%, respectively. Our smears were prepared directly from specimens without concentration, though Hooja et al. (2011) noted that concentrating specimens increases the sensitivity from 55.5% to 66.7%. The specificity of the ZN smear in the present study agreed with the study of Kang et al. (2016), who reported a specificity of 80.5%, with false positive results possibly due to the detection of dead bacilli, especially when considering that a proportion of the specimens were from patients under treatment which was the situation in our study. Accordingly, it was concluded that AFB smear alone is insufficient to assess treatment outcomes among TB patients (Hooja et al., 2011; Kang et al., 2016).

The GeneXpert assay has attributes of high sensitivity for MTB detection, as the detection limit was found to be 112.6 cfu/ml (Theron et al., 2014; Chakroverty et al., 2017). The higher sensitivity of GeneXpert compared to the ZN smear makes it very important in the rapid diagnosis of smear-negative patients with suspected TB with a low bacterial load, such as children and HIV-infected patients (Pinyopornpanish et al., 2015). In the present study, GeneXpert yielded an MTB positivity rate of 54%, with a sensitivity and specificity of 95% and 73.3%, respectively, compared to the gold standard L-J culture method. Additionally, its sensitivity increased to 100% for smear-positive specimens and decreased to 82.2% for smear-negative specimens. This is in strong concordance with the sensitivity and specificity of GeneXpert reported by several studies, with a higher sensitivity for smear-positive than smear-negative specimens (Pinyopornpanish et al., 2015; Albert et al., 2016; Shi et al., 2018).

In addition, Anyplex PCR detected MTB in 49 specimens, with a sensitivity and specificity of 95% and 81.7%, respectively, which is comparable to published data (Molina et al., 2015; Sali et al., 2016). Nonetheless, the slight variations in sensitivities and specificities

![Figure 3: Melting curves obtained using the Anyplex II MTB/MDR showing MDR-TB with RIF and INH resistance. Each curve is labeled as follows; IC: Internal control, MTB: MTB positive detection, RIF-R: rpoB mutation, INH-R1: katG mutation.](image-url)
for GeneXpert and Anyplex PCR between studies may be attributed to several factors: i) variable sample size, ii) different sample types (pulmonary/extra pulmonary), iii) presence of dead bacilli in tested specimens due to previous TB diagnosis and previous antituberculous drug intake, iv) and a variable bacillary load that may be low in certain type of patients such as those with HIV. Moreover, it was observed in the present study that both GeneXpert and Anyplex PCR had higher sensitivity than specificity, which was not reported in other studies (Matabane et al., 2015; Sali et al., 2016; Rice et al., 2017). According to Sali et al. (2016), this may be explained by the fact that specificity is usually higher than sensitivity in countries with a low TB burden but that the opposite is true in countries with a high TB burden (Sali et al., 2016). In the present study, the recorded low specificity was attributed to inclusion of samples for patients with a history of previous treatment.

Rifampicin resistance is considered a surrogate marker for MDR-TB because 90% of RIF-resistant isolates are associated with INH resistance (Molina-Moya et al., 2015). In our study, RIF’s resistance was detected in 3 of 54 MTB-positive specimens by GeneXpert, showing an MDR-TB rate of 5.5%. Anyplex PCR had an added privilege of being able to detect INH mono-resistance. Anyplex PCR detected resistance to both RIF and INH in 3 of 49 MTB positive samples, with an MDR-TB rate of 6.12% and was capable to detect INH mono-resistance in 6 MTB positive samples (12.2%). The rate of INH mono-resistance (12.2%) was near close to that reported by other studies (Sali et al., 2016; Rice et al., 2017). The MDR-TB rates recorded by GeneXpert and Anyplex assays in the present study are considered low compared to some studies that recorded higher rates (34.4%-53%) (Robledo et al., 2008; Mendoza et al., 2015; Igrashi et al., 2017). However, other studies complied with our study recording low MDR-TB rates of 1.5%-26% (Limaye et al., 2010; Anita et al., 2014; Kairwan et al., 2016; Rice et al., 2017). The variations in MDR-TB rates reported by several studies are attributed to several factors; i) variable TB burden geographically among different countries ii) different methods of detection, iii) different sample size iv) different study population being either, primary cases with no history of antituberculous drug intake or previously diagnosed cases who received treatment (Limaye et al., 2010; Mendoza et al., 2015). Both molecular assays showed significant agreement regarding susceptibility to RIF (P-value< 0.001); however, discrepant results were observed for 2 specimens. This discrepancy might be due to inadequate detection of Rif’s resistance in cases with hetero-resistance, with mixed populations of drug-resistant and drug-susceptible bacilli. Anyplex PCR was found to be incapable of detecting hetero-resistance being recognized as sensitive, as the mutated to wild-type ratio is not sufficiently high for detection (Andre et al., 2017; Dorman et al., 2018).

By comparing the performance with regard to MTB detection of the GeneXpert and Anyplex PCR assays in the present study, a categorical agreement of 89% was found for both. They also exhibited the same sensitivity of 95%, with a similar specificity of 73.3% for GeneXpert and 81.7% for Anyplex PCR. Both assays performed well when using smear-negative specimens, showing sensitivities of 88.2% and 94% for GeneXpert and Anyplex PCR, respectively, suggesting their strong utility in diagnosing cases with a low TB bacillary load (Matabane et al., 2015).

As referred to several studies, GeneXpert possess the following advantages: i) delivering results about both MTB detection and RIF resistance in less than 2 hours, ii) higher sensitivity than ZN smear microscopy, iv) high specificity with no false positive reactions by non tuberculous mycobacteria, v) it can be mediated for use in resource limited settings. Months) Nevertheless, few limitations were listed as: i) high cost, ii) short shelf life of cartridge (18 months), iii) the need to a very stable electric supply and temperature ceiling (Shrestha et al., 2015; Albert et al., 2016).

As development in the technical field does not stop, all Xpert limitations were considered by Cepheid to develop promising newer versions such as, Xpert MTB/RIF Ultra that was upgraded to overcome the limitations of Xpert MTB/RIF, up to the rise of GeneXpert® Omni which is a portable small device that provides accurate, and rapid point care testing for TB (Banada et al., 2016; Chakravorty et al., 2017).

In agreement with other studies, the present study demonstrated that GeneXpert had advantages over Anyplex PCR in rapid delivery of results within 2 hours, low contamination rates and technical ease of operation (Matabane et al., 2015; Sali et al., 2016). However, GeneXpert was limited by its cost, which is higher than that of Anyplex PCR, and its inability to detect INH mono-resistance. Anyplex PCR has relatively longer time assay, however, privileged by higher throughput capabilities than Xpert. Anyplex can test 96 specimens per run, unlike Xpert whose capacity is 4 specimens per run. Anyplex PCR is promising owing to its ability to detect both INH and RIF resistance and its lower cost, though it is limited by the requirement of highly trained staff and tendency for cross contamination (Matabane et al., 2015; Sali et al., 2016). The present study was limited by poor sample size that was due to financial barriers being conducted in a limited resource setting. However, it is recommended that every healthcare setting can assess the cost benefit of each assay and choose the optimum diagnostic method.

5. CONCLUSION

Our study showed that both GeneXpert and Anyplex PCR allowed rapid simultaneous MTB detection and drug susceptibility testing directly from clinical samples within 2 hours for GeneXpert and 3 hours for Anyplex PCR, with ease of operation. However,
GeneXpert has a higher cost than does Anyplex PCR. Both had the same sensitivity for MTB detection, which exceeds that of ZN smear; thus, these assays may be of particular importance for those suspected of having TB but with smear-negative results. Both assays showed a significant concordance in the genetic determinacy of resistance. Molecular methods offer strong advantages over conventional methods but cannot replace them. Each healthcare setting should choose which is most suitable with regard to workflow, cost, and applicability.

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Author Contributions
SMK, NGE, MMG designed the study and supervised research. MMB performed the practical work. RH and NSS had major contributions in analyzing results. NSS had the major contribution in writing the manuscript.

Funding
This study has not received any external funding.

Conflict of Interest
The authors declare that there are no conflicts of interests.

Ethical approval
The study was approved by the Medical Ethics Committee, Faculty of Medicine, Cairo University (ethical approval code: I-501015).

Informed consent
Written & Oral informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this manuscript.

Data and materials availability
All data associated with this study are present in the paper.

Abbreviations:
TB, Tuberculosis; MTB, Mycobacterium tuberculosis; MDR, multidrug resistant; WHO, World Health organization; L-J, Lowenstein Jensen; MGIA, mycobacterial growth inhibition assay; PCR, polymerase chain reaction; RIF, rifampicin; INH, isoniazid; NALC, N acetyl-L-cysteine; NAOH, sodium hydroxide; ZN, Zeil-neelsen; NTM, Non-tuberculous Mycobacterium; PNB, para-nitrobenzoic; RRDR, rifampicin resistance determining region.

REFERENCES AND NOTES