PCR-reverse blot hybridization assay in respiratory specimens for rapid detection and differentiation of mycobacteria in HIV-negative population

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Abstract

Objective: Rapid identification of pathogenic *Mycobacterium* species is critical for a successful treatment. However, traditional method is time-consuming and cannot discriminate isolated NTM to species level. In the retrospective study, we evaluated the clinical applicability of PCR-reverse blot hybridization assay (PCR-REBA) with clinical specimens for rapid detection and differentiation of mycobacterial species.

Methods: A total of 334 sputum and 362 bronchial alveolar lavage fluids (BALF) from 696 patients with mycobacterium pulmonary disease and 210 patients with non-mycobacterium pulmonary disease used as controls were analyzed. Sputum samples or BALF were obtained for MGIT 960 and PCR-REBA. To confirm mycobacterium species inconsistently identified by the two different assays, high resolution melting (HRM) analysis was performed.

Results: A total of 334 sputum and 362 BALF specimens from 696 patients with mycobacterium pulmonary disease (292 MTB and 404 NTM) were eventually analyzed. In total, 292 MTBC and 436 NTM isolates (co-infection of two species in 32 specimens) across 10 Mycobacterium species were identified. The most frequently isolated NTM species were *M. intracellulare* (n=236, 54.1%), *M. abscessus* (n=106, 24.3%), *M. kansasii* (n=46, 10.6%), *M. avium* (n=36, 8.3%). Twenty-two cases had mixed infection with both *M. intracellulare* and *M. abscessus* and ten cases had mixed infection with both *M. avium* and *M. abscessus*. A high level of agreement (n=696; 94.5%) was found between MGIT 960 and PCR-REBA (k = 0.845, P = 0.000). PCR-REBA had the higher AUC than MGIT 960 for both MTBC and NTM.

Conclusion: PCR-REBA is helpful for rapid mycobacterial species identification with low cost and simplicity and therefore recommending its routine use.

Introduction

Mycobacterium contains a number of acid-fast bacilli (AFB), including mycobacterium tuberculosis complex (MTBC), *Mycobacterium leprae*, and non-tuberculosis mycobacteria (NTM) (1). NTM are ubiquitous environmental organisms and cause progressive inflammatory lung damage, a condition termed ‘NTM pulmonary disease’ (NTM-PD) and extrapulmonary infections in susceptible individuals (2). With the prevalence of acquired immunodeficiency syndrome (AIDS), the recognition of clinical importance of NTM is also growing (3). Many countries in the world have recently reported a dramatically increased incidence of pulmonary NTM-positive cultures (4, 5, 6,7). NTM infections constitutes 0.5–35% of all human mycobacterial infections, while it is more common in individuals with chronic lung diseases such as bronchiectasis, cystic fibrosis, and immune deficiency (8). This rate approaches 50% in patients with risk factors (9). However, a high percentage of patients have no risk factors (10). Infections of most NTM species have traditionally been considered from environmental sources rather than person-to-person transmission like MTBC (11). In addition, many NTM strains are insensitive to most anti-tuberculosis drugs, and the therapeutic regimens differ significantly from that of MTBC (12, 13). Therefore, rapid and

accurate identification of mycobacterium species is necessary for effective therapy and infection control strategy (14, 15). However, MTBC and NTM infections exhibit similar clinical symptoms (cough, sputum production, fatigue, hemoptysis and fever) associated with smear-positive samples, that frequently cause diagnostic uncertainty. Abnormalities seen on CT, such as centrilobular nodules, tree-in-bud opacity and cavitation, cannot reliably distinguish between infections caused by TB and NTM, or between infections caused by different species of NTM. The traditional methods used in mycobacterial laboratories require pure isolates, which delays many months to obtain results. This delay impacts clinical management of the patient and potentially prolongs treatment course. These methods are time-consuming, labor-intensive, and often not definitive and associated with additional costs that are not always considered (16). Furthermore, these assays could not discriminate isolated NTM to species level, or the mixed infection, in which two or more mycobacterial species were identified simultaneously from the same specimen (17). All NTM isolates from respiratory samples should be identified to at least species level, since that optimal treatment regimens differ by species, with major differences between slow-growing and rapid-growing species. Therefore, these methods are not conducive to guide clinical decisions in a timely manner. Clinicians sometimes can only refer to interferon-γ release assays (IGRAs), which are based on M. tuberculosis specific antigen. These specific antigens are not present on NTM strains other than M. marinum, M. kansasii, M. gordonae and M. szulgai (18). In addition, it is worth mentioning that IGRAs is negative in a number of TB patients (19). So, IGRAs cannot reliably distinguish NTM from *M. tuberculosis.*

In the last decade, advances in molecular assays have accelerated the reliable diagnosis of mycobacterial infections. Among these methods, nucleic acid amplification (NAA)-based techniques permit a higher level of discrimination on mycobacterial species (20-23). Sequencing of 16S rRNA has been recommended as the gold standard method for definitive identification and discrimination of mycobacterial species (24). PCR-reverse blot hybridization assay (PCR-REBA), based on 16S rRNA sequencing and nucleic acid probes and reverse hybridization, has been established for the genotyping of 22 clinical important mycobacterial species including *MTB, M. smegmatis, M. Intracellulare, M. kansasii, M. chelonae, M. marinum, M. fortuitum, M. terrae, M. nonchromogenicum, M. Avium, M. scrofulaceum, M. abscessus, M. xenopi, M. gilvum, M. phlei, M. triviale, M. gordonae, M. gastri, M. vaccae, M. szulgai, M. diernhoferi, M. simiae*. Moreover, as a major advantage, PCR-REBA can be performed directly using clinical specimens. However, this method is not incorporated into the workflow of many mycobacterial laboratories, and whether it can replace the traditional methods in clinical practice remains to be supported by clinical large-sample-size studies. Therefore, the purpose of this retrospective single-center study was to highlight the clinical applicability of PCR-REBA for rapid detection and differentiation of mycobacterial species from a sizable cohort of patients compared with BACTECTM MGIT 960 culture (MGIT 960) and biochemical tests in the high-burden settings of China.

Materials And Methods

Ethics Statement and Informed Consent

The study was performed according to the Declaration of Helsinki with respect to ethical principles for research involving application of human specimens. Written informed consent for a retrospective-review protocol approved by The Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine in China had been provided by all of the patients.

Clinical specimen collection

All patients with clinically suspected NTM-PD attending Shanghai Pulmonary Hospital between January 3, 2018, and November 28, 2019, were eligible for screening if they met the following inclusion criteria: 1) negative for HIV test; 2) providing sputum specimens for examinations, or bronchial alveolar lavage fluids (BALF) from sputum-scarce patients. Two sputum samples or BALF were obtained for MGIT 960 and PCR-REBA at screening. Respiratory samples should be processed within 24 hours of collection (or refrigerated at 4°C if delays are anticipated).

Fiberoptic bronchoscopy

Bronchoscopy procedures were performed according to our institute's infection regulation and manufacturer's instruction guidelines (BF-1 T260, Olympus, Tokyo, Japan). Inspectors wore N95 masks, goggles and gowns during the procedure. The bronchoscopy room was equipped with negative pressure isolation and an air disinfection system.

Routine Identification Methods

MGIT960 culture: Each specimen of approximately 5 ml was decontaminated using the Nacetyl-L-cysteine (NALC)–NaOH method (25). The final concentration of NaOH is 4%. The samples exposed to NaOH for 15–20 min. The processed sediment was washed once using a sterile 0.9% NaCl solution and resuspended in 1.5 ml sterile 0.9% NaCl solution; two separate 500-μl aliquots were prepared in 1.5-ml tubes for PCR-REBA and MGIT 960 culture. Specimens were cultured by MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD) for 6 weeks following the standard procedure of the manufacturer (26). Then samples from positive signaled MGIT 960 tubes were Ziehl-Neelsen (ZN) -stained and Gram-stained to confirm the presence of mycobacteria and exclude contamination.

TBc identification test (TBc ID**):** TBc ID (Becton Dickinson, Sparks, MD) is an immunochromatographic assay for the detection of MPT64 Ag, which is a mycobacterial protein secreted only by MTBC and has been shown to differentiate MTBC from NTM. 100-μl of liquid media from the positive MGIT tubes was added to the TBc ID card. Then the cards were incubated for 15 min at room temperature and the results were visually assessed. A positive test result indicated MTBC while a negative test result indicated NTM.

Para-nitrobenzoic acid (PNB) test: The PNB media was prepared locally, stored between 2 °C – 8 °C and quality controlled according to our standard operating procedures. The test required two Lowenstein Jensen slants one with and another without PNB reagent, the latter serving as negative control. Both slants were inoculated with 500 μL of liquid media from the negative MPT64 Ag tubes, incubated at 37 °C

and read weekly until colonial growth was observed. Identification of *NTM* was made based on presence of growth in the tube with PNB, with no growth in the control tube (without PNB).

PCR-reverse blot hybridization assay (PCR-REBA): a) DNA Isolation: 500 µl bacterial precipitate were treated with DNA Lysis Buffer (10 mmol/L NaCl, 1 mg/ml SDS, 0.15 g/ml Chelex-100 glass beads, 1% Tween 20) at 50°C for 1 hour, then at 100°C for 10 minutes, and centrifuged at 10000r/min for 2min. The supernatant containing genomic DNA was transferred into new tube and stored at -20°C for PCR amplification. b) Amplification: PCR was carried out in a 50µl reaction mixture containing 5 µl DNA template, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTP, 0.4 mM each primer, and 2 U AmpliTaq Gold polymerase. The mixture was first incubated at 94°C to activate the Taq polymerase, followed by 40 cycles of amplification (94°C for 1 minute, annealing and extension for 30 seconds at 65°C, 72°C for 1 minute), and finally 72°C for 10 minutes. A 5µl aliquot of the PCR product was electrophoresed on 6% polyacrylamide gel with silver staining. c) Hybridization: The amplified PCR products were then subjected to REBA using Mycobacterium species identification detecting Kit (Yaneng BioSciences, Shenzhen) according to the manufacturer's instructions. In brief, biotinylated PCR products were denatured at 25°C for 5min and added to the REBA membrane strip in the provided blotting tray. Denatured single-stranded PCR products were hybridized with the probes on the strip at 55°C for 30min. The strips were then washed twice with gentle shaking in 1.0ml of washing solution for 10min at 55°C, incubated at 25°C for 30min, and washed twice with 1.0ml of CDS at room temperature for 1min. Finally, the colorimetric hybridization signals were visualized and the band pattern was read.

High Resolution Melting (HRM) analysis: To confirm mycobacterium species inconsistently identified by the two different assays, fluorescence PCR-HRM Assay was performed using Mycobacterium Identification Kit (Zeesan Biotech, Xiamen). The amplification of rpoB gene was performed on the following conditions: a pre-incubation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 65°C for 30 s, and extension at 72°C for 10 s followed by the Tm analysis with increasing temperatures from 60 to 95°C in a 0.2°C s-1 slope increment for 10 s. The HRM analysis was performed using Gene Scanning Software Version 1.5.0 (Roche Instrument Centre, Switzerland). The clustering of the melting curves was based on the regions of the melting curve corresponding to the pre-melting, melting, and post-melting regions. Distilled water was used instead of the DNA template as the non-template control (NTC).

Statistical Analysis

SPSS for Windows (Version 19.0, SPSS Inc., Chicago) was used for data analysis. The patients' characteristics and detection results were integrated together. All patients were followed up for at least 6 months with assessment of symptoms, radiological change and mycobacterial culture results. We calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of PCR-REBA assay and MGIT 960- TBc ID-PNB test. The categorical variables were analyzed using Fisher exact or Pearson X^2 tests where appropriate and 2-tailed tests were used. The concordance of agreement between MGIT 960- TBc ID-PNB test and PCR-REBA assay was evaluated using Cohen's kappa test (k >

0.75, excellent agreement; 0.4 < k < 0.75, moderate agreement; and k < 0.4, poor agreement). Receiver operating characteristic (ROC) curve analysis was performed to determine the power of MGIT 960- TBc ID-PNB test and PCR-REBA assay to distinguish PTB and NTM patients from non-mycobacterium patients. The area under curve（AUC）with a 95% confidence interval (CI) was further calculated. P <0.05 was considered statistically significant.

Results

Demographic and Clinical Characteristics of the Participants

A total of 1378 clinical specimens (698 sputum and 680 BALF) from 1378 patients with clinically suspected NTM-PD were subjected to PCR-REBA and MGIT 960 culture. Finally, 682 patients were excluded, including 383 patients with obscure diagnosis, 89 patients lost to follow up and 210 patients with non-mycobacterium pulmonary disease (Table 1). The remaining 334 sputum and 362 BALF specimens from 696 patients with mycobacterium pulmonary disease (292 MTB and 404 NTM) were eventually analyzed (Figure 1). Baseline characteristics of the 696 mycobacterium pulmonary disease patients were summarized in Table 2. In total, 292 MTBC and 436 NTM isolates (co-infection of two species in 32 specimens) across 10 Mycobacterium species were identified. The most frequently isolated NTM species were *M. intracellulare* (n=236, 54.1%), *M. abscessus* (n=106, 24.3%), *M. kansasii* (n=46, 10.6%), *M. avium* (n=36, 8.3%), M.scrofulaceum (n=4, 0.9%), M. phlei (n=2, 0.5%), M.chelonae (n=2, 0.5%), *M. xenopi* (n=2, 0.5%), and *M. marinum* (n=2, 0.5%). Twenty-two cases had mixed infection with both *M. intracellulare* and *M. abscessus* and ten cases had mixed infection with both *M. avium* and *M. abscessus* (Table3).

Identification of Mycobacterial by MGIT 960- TBc ID-PNB test

Of the 292 pulmonary tuberculosis (PTB) patients, 274 isolates (93.8%) were identified as MTBC and 10 were negative by MGIT 960. Of the 404 NTM-PD patients, 394 isolates (96.5%) were identified as NTM and 6 were negative by MGIT 960 (Table 4). Since two false-positive MGIT 960 cultures in the non-TB group were identified as MTBC and NTM, the specificity of MGIT 960 test for both MTBC and NTM was 99.5% Eight MTBC strains were incorrectly identified as NTM, and four NTM strains were incorrectly identified as MTBC by MGIT 960.

Identification of Mycobacterial by PCR-REBA

Of the 292 PTB patients, 286 isolates (97.9%) were identified as MTBC and 6 isolates were negative by PCR-REBA assay. Of the 404 NTM-PD patients, 400 isolates (99.0%) were identified as NTM and 4 isolates were negative by PCR-REBA assay. PCR-REBA assay demonstrated 100% of specificities and PPVs for MTBC and NTM. The PPVs of PCR-REBA assay for both MTBC and NTM were significantly higher than that of MGIT 960 (P=0.04). The sensitivity of PCR-REBA assay for MTBC was significantly higher than that of MGIT 960 (P=0.012).

Consistency between MGIT 960- TBc ID-PNB test and PCR-REBA

MGIT 960 test and PCR-REBA were completely concordant except for 38 samples (5.5%) (Table 5). For the 38 strains with inconsistent results of the two methods, HRM method was used for further analysis. A high level of agreement (n=696; 94.5%) was found between MGIT 960 and PCR-REBA (k = 0.845, P = 0.000), indicating an optimal consistency between the 2 tests.

Establishment of ROC curve

The area under ROC curve (AUC) of PCR-REBA and MGIT 960 was 0.990 (95% CI 0.980–0.999) and 0.964 (95% CI 0.947–0.982) for MTBC, 0.995 (95% CI 0.988–1.0) and 0.983(95% CI 0.971–0.994) for NTM (Figure 2, 3). PCR-REBA had the higher AUC than MGIT 960 for both MTBC and NTM.

Discussion

In this retrospective study, we evaluated the clinical applicability of PCR-REBA for rapid detection and differentiation of mycobacterial species directly using respiratory samples in patients with clinically suspected NTM-PD and found that PCR-REBA assay has higher sensitivity and PPV than automated liquid culture systems, which is consistent with the results of the previous study by Lee et al. (27). As NTM are ubiquitous environmental organisms, a minimum of two sputum samples collected on separate days should be sent for mycobacterial culture when investigating an individual suspected of having NTM-PD. A single NTM isolate from sputum, which is not isolated again on repeated culture, is usually of no clinical relevance. Because of the slow growth rate of mycobacteria, the differentiation of Mycobacteria to the species level by evaluation of phenotypic and biochemical testing is time-consuming. However, the major advantage of direct molecular detection is to rapidly differentiate M. tuberculosis complex from NTM in clinical samples. PCR-REBA has a short turnaround time of about 4 hours. Achieving NTM eradication aiso require earlier intervention. Another limitation of mycobacterial liquid culture is that the accuracy is greatly influenced by sample quality, mycobacterial species and laboratory expertise. Relatively high contamination rate was reported (28). Our results showed that the overall contamination rate was 1.7% (12/696). In addition, there were two false positive cultures in the non-TB group including one MTBC and one NTM isolate. Culture of NTM can be difficult even in mycobacterial reference laboratories. Routinely culture is performed at 35°C, but many NTM species have optimum growth at 28°C–30°C or 45°C. Some NTM species require supplemented media or extended duration of incubation. In our current study, six strains of NTM were not cultured, but were detected by PCR-REBA and confirmed by HRM analysis. Routinely culture also cannot discriminate NTM species. At present, more than 160 distinct *Mycobacterium* species have been validly published (http://www.bacterio.net/mycobacterium.html). The clinical relevance of isolated NTM differs strongly by species, from pathogenic species (e.g., *Mycobacterium kansasii* and *Mycobacterium malmoense*) to typical saprophytes (*Mycobacterium gordonae* and *Mycobacterium phlei)* (29). In practice, treatment recommendations presented for NTM are based on the NTM species. NTM isolates species should be subspeciated. For example, disease due to M. avium was associated with a worse prognosis than that

attributable to *M. intracellulare,* and patients with *M. massiliense* tend to have better treatment responses than those with *M. abscessus* (30). However, PCR-REBA cannot further classify *M. abscessus* as *M. massiliense*, *M. bolletii* and *M. abscessus* subspecies. If person-to-person transmission of *M. abscessus* is suspected, isolates should be typed, preferably using whole genome sequencing (WGS), a new technology of the last few years. However, this technique is not cost-effective and require expensive equipment.

Conclusion

To conclude, PCR-REBA is an efficient tool and has higher specificity and rapidity than conventional methods. If there is high clinical suspicion of NTM infection, the utility of molecular detection methods provides a useful adjunct to culture and should be routinely carried out to identify all NTM isolates to at least species level to facilitate rapid selection of the appropriate therapeutic regimens. Furthermore, it does not require specialized instruments and costs less than other non-culture-based methods, resulting in an affordable alternative method in source-limited countries.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

LY, QZ and HX were responsible for the conception and design of the study. LY, QZ and HX were responsible for acquisition and analysis of data; furthermore, LY was in charge of statistical analysis. LY took part in drafting the manuscript; LY, QZ and HX revised and approved the final version of the manuscript. All authors read and approved the final submitted version.

Ethics declarations

Written informed consent for a retrospective-review protocol approved by The Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine in China had been provided by all of the patients.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. Baseline characteristics of patients with non-mycobacterium pulmonary disease.

Condition	Age±SD (range)	Gender (male: female)	MGIT 960 (+)	PCR-REBA (+)	Total (210)
Lung cancer	48±16 (35-80)	49:38	0	0	87
Pneumonia	49±17 (21-78)	35:30	1(NTM)	0	65
Pulmonary mycosis	51±17 (35-80)	15:13	0	0	28
Silicosis	48±8 (40-57)	20:1	0	0	21
Bronchogenic cyst	49±4 (45-51)	1:1	0	0	2
Interstitial lung disease	50±2 (48-52)	1:1	0	0	2
Pulmonary embolism	50±1 (49-51)	1:0	1(MTBC)	0	1
Granulomatous vasculitis	43	0:1	0	0	1
Right lower lobe sequestration	46	1:0	0	0	1
Lung tissue X disease	51	1:0	0	0	1
Myelodysplastic syndrome	45	0:1	0	0	1

Table 2. Baseline characteristics of patients with mycobacterium pulmonary disease.

	MTB pulmonary disease (n=292)	NTM pulmonary disease (n=404)
Age, SD (range)	44±15 (25-80)	49±17 (32-86)
Gender (male: female), n	190:102	191:213
Body mass index, median (range)	19.3 (14–29)	19.2 (13–28)
Diabetes mellitus, n	32	21
Autoimmune diseases, n	18	25
bronchiectasis or cystic fibrosis	101	98
Smear (+) / QFT (-)	73	240
No response to anti-TB medication	118	66

Table 3. Identification of clinical isolates using PCR-REBA

Clinical organism	Number of strains (728)	Identified by PCR-REBA (718)
MTB	292 (40.1%)	286 (97.9%)
M. intracellulare	236 (32.4%)	232 (98.3%)
M. abscessus	106 (14.6%)	106 (100%)
M. kansasii	46 (6.3%)	46 (100%)
M. avium	36 (4.9%)	36 (100%)
M.scrofulaceum	4 (0.5%)	4 (100%)
M. xenopi	2 (0.3%)	2 (100%)
M. marinum	2 (0.3%)	2 (100%)
M. phlei	2 (0.3%)	2 (100%)
M.chelonae	2 (0.3%)	2 (100%)

Table 4. Comparison of PCR-REBA and MGIT 960 for Detection of mycobacterium

		PCR-REBA	MGIT 960	*P*
MTB	sensitivity	97.9%	93.8%	0.012
	specificity	100%	99.5%	0.317
	PPV	100%	98.6%	0.040
	NPV	98.0%	96.7%	0.323
NTM	sensitivity	99.0%	96.5%	0.106
	specificity	100%	99.5%	0.317
	PPV	100%	98%	0.004
	NPV	99.0%	98.5%	0.530

Table 5. Identification of clinical isolates using PCR-REBA, MGIT 960 and HRM

PCR-REBA	MGIT 960	HRM	Total (696)
MTB	-	MTB	10
MTB	NTM	MTB	8
-	MTB	MTB	6
M.scrofulaceum	MTB	M.scrofulaceum	4
-	NTM	M. intracellular	4
M. intracellular	-	M. intracellular	2
M. phlei	-	M. phlei	2
M.chelonae	-	M.chelonae	2
MTB	MTB	/	268
M. intracellulare	NTM	/	208
M. abscessus	NTM	/	74
M. kansasii	NTM	/	46
M. avium	NTM	/	26
M. xenopi	NTM	/	2
M. marinum	NTM	/	2
M. intracellulare-M. abscessus	NTM	/	22
M. avium-M. abscessus	NTM	/	10

Figures

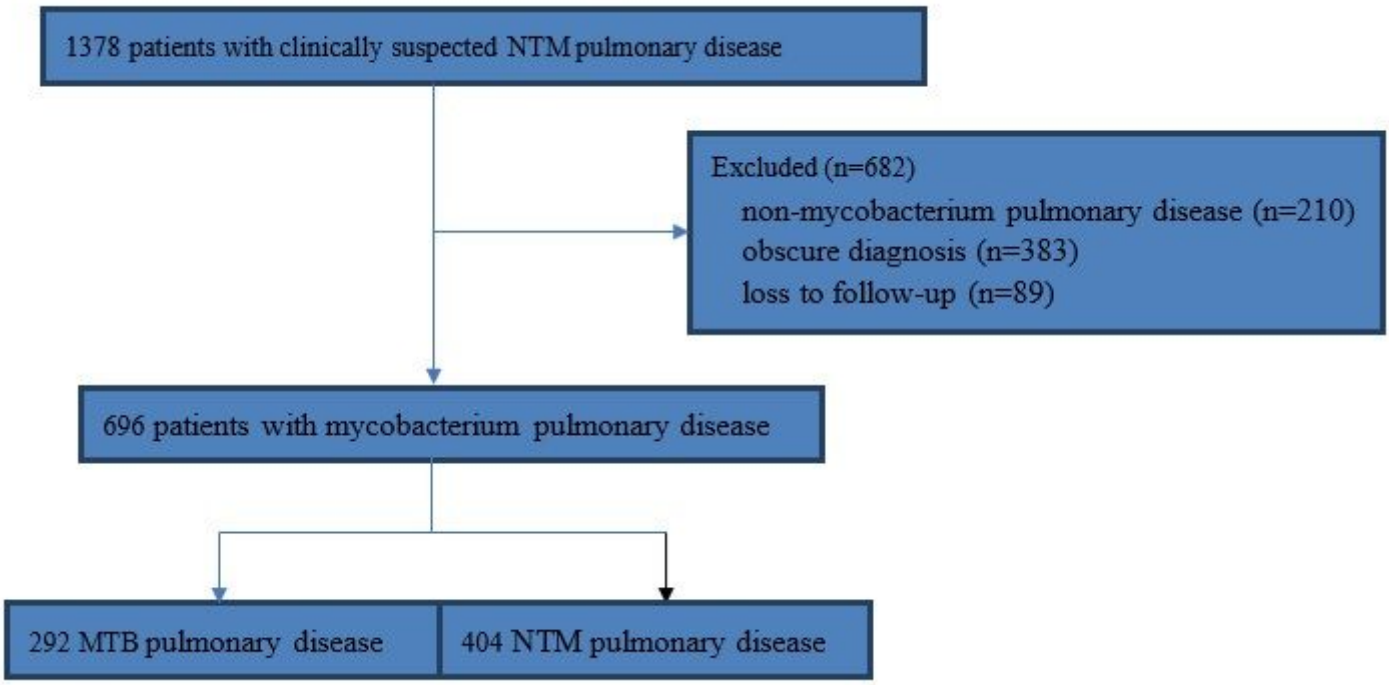

Figure 1

Flow chart of the study.

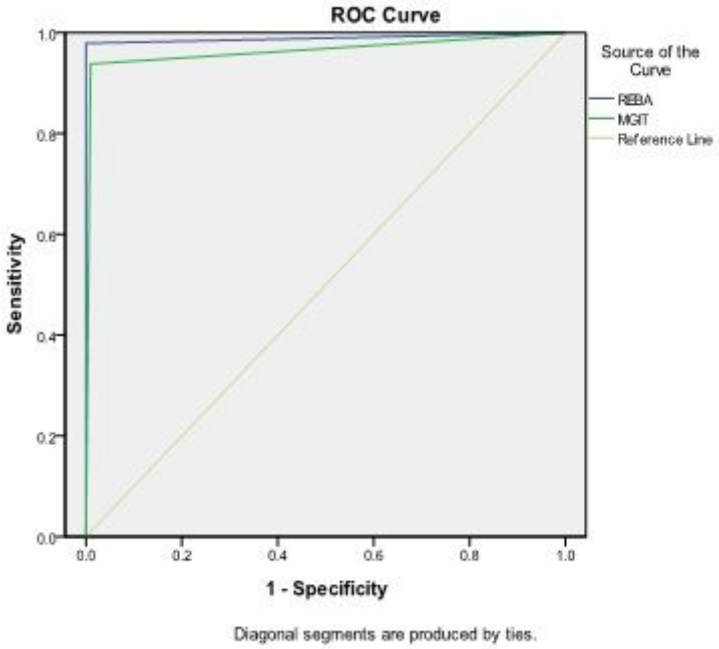

Figure 2

ROC curve of REBA and MGIT 960 for MTBC

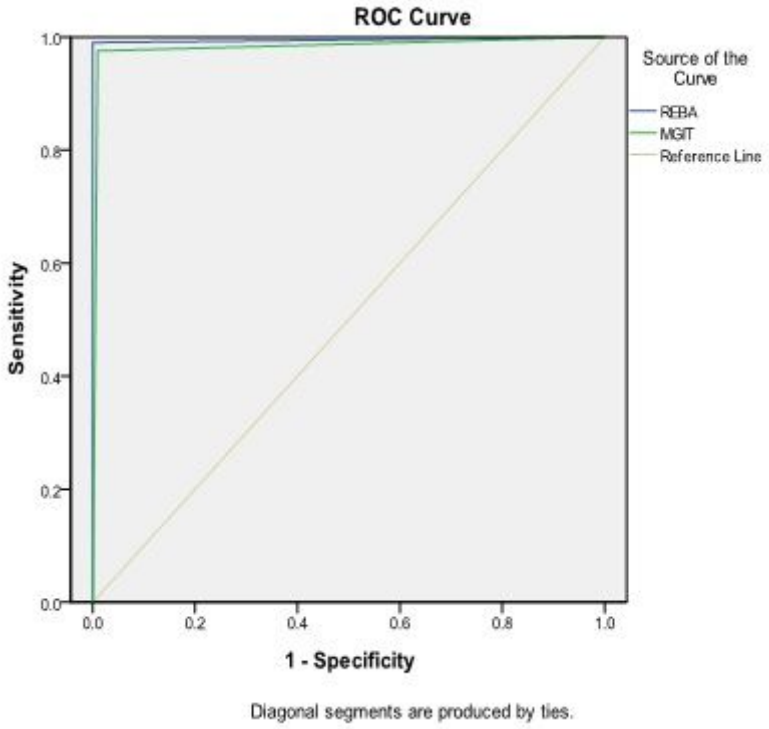

Figure 3

ROC curve of REBA and MGIT 960 for NTM