Gene Xpert MTB/RIF Assay as A New Tool for Tuberculosis Diagnosis and Detection of Rifampin Resistance

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Abstract

Background: Mycobacterium tuberculosis (MTB) is one of the top ten causes of death and the leading cause from a single infectious agent (above HIV/AIDS) Worldwide. Each year millions of people continue to fall sick with TB. Aim: To evaluate the MTB/RIF assay for rapid diagnosis and detection of rifampin resistance of tuberculosis in both positive and negative smears as well as pulmonary and non-pulmonary clinical specimens. Patients and Methods: A prospective study was conducted on 50 patients (37 pulmonary, 13 extrapulmonary) patients attending the Outpatient Clinic and inpatients of Chest department, Benha University Hospital, Egypt. Pulmonary samples were analyzed by (Sputum smear exam. by Ziehl Neelsen (ZN) stain, culture on Lowenstein-Jensen (LJ) media, and assessment of rifampin sensitivity). Extrapulmonary samples were analyzed by reference methods (pathological diagnosis) and by GeneXpert. Results: pulmonary patients’ smears were 32 (86.5%) positive and 5 (13.5%) negative. Culture on LJ revealed rifampin sensitivity in 33/37 (89.1%) and rifampin resistant in 4/37 (10.9%), GeneXpert was positive in 32(86.5%) and negative in 5 (13.5%), rifampin assay was sensitive in 33 (89.1%) and resistant in 4 (10.9%). All extrapulmonary patients, (n=13) were positive on GeneXpert, and sensitive for rifampin. Conclusion: GeneXpert could be considered as a leading way to early diagnosis, treatment, and prevention of transmission of tuberculosis which could reduce TB- associated morbidity and mortality.

Key words: Ziehl-Neelsen Smears, Lowenstein-Jensen media, and Rifampin sensitivity

Introduction

Tuberculosis (TB) remains the world’s deadliest infectious killer. Each day, nearly 4500 people lose their lives to TB and close to 30,000 people fall ill with this preventable and curable disease. Global efforts to combat TB have saved an estimated 54 million lives since the year 2000 and reduced the TB mortality rate by 42%. To accelerate the TB response in countries to reach targets – Heads of State came together and made strong commitments to end TB at the first-ever UN High Level Meeting in September 2018¹. Microscopy, culture, and drug susceptibility testing (DST) are standard methods used in laboratory diagnosis of TB but they are time-consuming processes, taking up to eight weeks for diagnosis. Doctors in the developing world often rely only on chest X-rays without referring patients for sputum smears; confirmation by culture is not done routinely. Moreover, diagnosis of extrapulmonary TB

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is difficult to establish due to the low number of bacteria in clinical specimens. Rapid and accurate diagnosis of pulmonary and extrapulmonary TB is still a great challenge in developing countries due to limited resources and a lack of laboratory expertise\(^2\). Therefore, new developments in molecular diagnostics have been introduced into practice, playing a pivotal role in early diagnosis of and prompt response to TB. Advances in molecular diagnostics, drugs, and vaccines have improved chances for better TB control at a global level\(^3\). Several molecular methods have been developed during the last decade for TB diagnosis and rapid detection of TB resistance, including line probe assays GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) and INNO LIPA Rif.TB nnogenetics, Ghent, Belgium), and real-time polymerase chain reaction (PCR) GeneXpert MTB/RIF (Cepheid, Sunnyvale, USA)\(^2\). The GeneXpert MTB/RIF assay is a hemi-nested real-time PCR assay for the diagnosis of TB as well as rapid detection of rifampin (RIF) resistance in clinical specimens within two hours\(^4\). The GeneXpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and heminested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens\(^5\). The MTB/RIF assay detects \(M.\) \(tuberculosis\) and RIF resistance by PCR amplification of the 81-bp fragment of the \(M.\) \(tuberculosis\) \(rpoB\) gene and subsequent probing of this region for mutations that are associated with RIF resistance. The assay can generally be completed in less than 2 hours\(^6\). The current study aimed to evaluate the MTB/RIF assay for rapid diagnosis and detection of rifampin resistance of tuberculosis in both positive and negative smears as well as pulmonary and non-pulmonary clinical specimens.

### Patients and Methods

A prospective study was conducted on 50 patients attending the Outpatient Clinic and inpatients of Chest department, Benha chest Hospital and Ismailia Chest hospital in the period from December 2018 to May 2019. Laboratory tests were carried out at the Microbiology unit of clinical pathology department of Benha University Hospital. The study included two groups of patients: i) Patients with pulmonary TB were 37 cases: 30 under treated patients and 7 newly diagnosed. ii) patients with non-pulmonary tuberculosis \((n=13)\) included: lymph node TB \((n=5)\), TB pleural effusion, TB peritoneal fluid, TB of bones \((n=2\) each), TB kidney and skin TB \((n=1\) each). All patients were subjected: Full history taking; Clinical examination, radiological examination, Complete Blood Count, blood urea, serum creatinine. ALT, AST, FBS, ESR. Two sputum samples were obtained from each patient (one for GeneXpert and the other for conventional methods). For conventional methods: sputum samples underwent decontamination, processing, Ziehl Neelsen staining, culture on Lowenstein-Jensen (LJ) medium, and assessment of Rifampin sensitivity. Non-Pulmonary cases: specimens were collected according to their sites each sample was divided into 2 parts one for pathological diagnosis and the other for GeneXpert.

**Specimen collection:**

A. Pulmonary cases: (Sputum samples):

Early morning sputum samples were collected by asking the patient to cough deeply in a sterile wide screw capped container. The cap of the container is tightly sealed, and its outer surface is wiped off with disinfectant solution in a cotton swab. Name, age, and date were written obviously on containers, held in the refrigerator to inhibit the growth of contaminating organisms until reaching Microbiology Lab. Specimens were transported within 48 hours to the laboratory. Each sample was divided into 2 parts one for Microbiology Lab and the other for GeneXpert. B. Non-Pulmonary specimens were collected according to the sites (lymph node, skin, bones and kidney by incisional biopsies;
and pleural/peritoneal by aspiration techniques. Each sample was divided into 2 parts one for pathological diagnosis and the other for GeneXpert.

**Bacteriological examination of sputum specimens**

All techniques were done under complete aseptic techniques and inside biological safety cabinet. 1) **Decontamination** was performed by Petroff’s Method and Modified Petroff’s Method. 2) **Ziehl Neelsen Staining**: the presence of any red bacilli were reported as ‘AFB positive’ smear (More than 10 AFB/field in at least 20 fields was reported as (+++), 1-10 AFB/field in at least 50 fields was reported as (++), 10-99 AFB/100 fields was report as (+), 1-9 AFB/100 fields was reported as the exact number). 3) **Culture** this was performed using LJ medium, reading culture started 2 weeks after cultures up to 8 weeks: The time between inoculation and the earliest reading when colonies resembling mycobacteria were noted on the agar surface. Specimens were considered culture negative if there were still no colonies formed after 8 weeks of incubation. Culture was identified by: 1) **Rate of growth**: The organisms that grow after 7 days incubation were considered slow growers. 2) **Macroscopically**: colonies were buff colored and rough, having the appearance of breadcrumbs or cauliflower (interpretation: 0:= Number of colonies less than 20, +:= 20-100 colonies, ++= Numerable colonies but growth not confluent, +++=Confluent growth. 3) **Microscopical identification by Ziehl Neelsen Staining**, 4) **PNB test**: Pyrazinamidase test growth on P-Nitrobenzoic acid showed No growth, 5) **TCH test**: Thiophene-2-carboxylic acid hydrazide showed growth.

**Rifampin Susceptibility test**: A suspension was prepared: 1 ml sterile distilled water with six of (3mm) glass beads and 1 one loop full (3 mm internal diameter) of culture, vortexed for 20 – 30 seconds then 4 ml of SDW was added to the above and adjusted turbidity with McFarland 1 % with SDW. For Inoculation for Rifampin, 2-3 drops from the bacterial concentration prepared above were added to the LJ media containing Rifampin previously prepared. In all media slopes were incubated at 37°C and placed in the incubator in chronological order. The inoculated bottles were incubated at a horizontal position for 24-48 hours to ensure even distribution of the inoculum. After that, the incubation of the tube was continued in an upright position for 28 days at the same temperature. A control bottle for each positive growth is made on LJ media only parallel with sensitivity bottle.

**Test Interpretation**

First reading was taken at 28th (not after that) day after inoculation. The colonies were counted only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). If the result on the 28th day was “resistant”, no further reading of the test for that drug was required and the strain was classified as resistant. If the result at the 28th day was “sensitive”, a second reading was made on the 42nd day only for the sensitive strain. The final definitive results for Rifampin was reported on 42nd day. In case growth on the control media was poor even after six weeks (i.e., few or no colonies on the 10-4 bacterial dilution), the test was repeated.

**GeneXpert testing:**

**Specimen processing**: The Xpert MTB/RIF assay was used for sputum samples after decontamination and can be used directly for homogenized extrapulmonary samples (lymph node biopsies and other tissues) or on decontaminated specimens if culture was performed concurrently. Whenever possible, specimens were transported and
stored at 2 to 8°C prior to processing (a maximum of 7 days).

**Steps for GeneXpert testing**

a. Half ml of re-suspended sediment was transferred to a conical screw-capped tube, 1.5 ml of Xpert MTB/RIF sample reagent was added by sterile pipette, and the tube was recapped and shaken vigorously 10–20 times. b. The sample was incubated for a total of 15 minutes at 20–30°C, with manual agitation 10–20 times at one point between 5 and 10 minutes into the incubation period. c. The reagent-treated sample was then transferred by sterile pipette into the sample chamber of the Xpert MTB/RIF cartridge and loaded into the GeneXpert Dx instrument system for sample processing.

**Interpretation of the GeneXpert test**

i) In the event of “no result”, “invalid” or “error” results, test was repeated. ii) Positive for TB and Rifampin sensitive, iii) Positive for TB and Rifampin resistant, iv) Negative for TB.

**Statistical analysis**

The data were recorded on an “Investigation report form”. These data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 16. Descriptive statistics were calculated for the data in the form of Mean, Number and percentage. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated to detect the diagnostic power of different used test methods in comparison to the reference method. P value <0.05 was considered statistically significant.

**Results**

This study was performed on 50 cases (pulmonary (n=37) and extrapulmonary (n=13)) tuberculous patients. 37 pulmonary patients and 13 extrapulmonary patients.

**Pulmonary cases**

ZN staining was positive in 32 cases (86.5%) while 5 cases (13.5%) were negative; (Table 1). For GeneXpert: 32 cases (86.5%) were positive and 5 cases (13.5%) were negative. The 5 negative cases for ZN were also negative for GeneXpert (Table 1). Sensitivity and specificity of ZN stain were 86.5% and 100% respectively; PPV and negative predictive value 100% and 80% respectively and accuracy 91%. Sensitivity and specificity of GeneXpert was 86.5% and 100% respectively; PPV and NPV 100% and 80% respectively and accuracy 91% (Table 2). Rifampin manual in pulmonary cases showed that 33 (89.1%) were sensitive and 4 (10.9%) were resistant, Rifampin by GeneXpert in pulmonary cases showed that 32 cases (86.5%) were sensitive and 5 cases (13.5%) were resistant (Table 3). In extrapulmonary cases (diagnosed for TB by pathological biopsies); both GeneXpert and Rifampin by GeneXpert were positive in all the cases (100% each). GeneXpert assay in all studied Tuberculosis cases (pulmonary and extra pulmonary) showed 45 cases (90%) positive and 5 (10%) negative (Table 4). The performance of Gene Expert in detection of all studied patients is shown in table 5. sensitivity and specificity in all patients were 100% and 90% respectively; PPV and NPV 100% and 80% respectively and accuracy 92.8%. Sensitivity and specificity of GeneXpert in pulmonary patients were 86.5% and 100% respectively; PPV and NPV were 100% and 80% respectively and accuracy 91%. Sensitivity and specificity of GeneXpert in extra pulmonary were 100% and 100% respectively; PPV and NPV (100% each) and accuracy 100%. Rifampin sensitivity by GeneXpert in all TB cases is
demonstrated in table 6; 45 cases (90%) were sensitive and 5 cases (10%) were resistant.

**Table 1:** Comparison between ZN stain, LJ media and gene expert in cases with pulmonary TB patients

| Variation | Number | Percent |
|-----------|--------|---------|
| ZN Positive | 32 | 86.5% |
| ZN Negative | 5 | 13.5% |
| LJ Positive | 37 | 100.0% |
| LJ Negative | 0 | 0.0% |
| GeneXpert Positive | 32 | 86.5% |
| GeneXpert Negative | 5 | 13.5% |

**Table 2:** The performance of ZN, LJ and GeneXpert in detection of pulmonary studied patients.

|          | Sensitivity | Specificity | PPV   | NPV   | Accuracy |
|----------|-------------|-------------|-------|-------|----------|
| ZN       | 86.5%       | 100.0%      | 100.0% | 80.0% | 91.2%    |
| LJ       | 100.0%      | 100.0%      | 100.0% | 100.0%| 100.0%   |
| GeneXpert| 86.5%       | 100.0%      | 100.0% | 80.0% | 91.2%    |

**Table 3:** Comparison between Rifampin sensitivity by conventional method (manual) and by GeneXpert in pulmonary TB

|          | Pulmonary TB | N=37 |
|----------|--------------|------|
| Gene expert Rifampin | Sensitive | 32 | 86.4% |
| Gene expert Rifampin | Resistant | 5 | 13.6% |
| Manual Rifampin | Sensitive | 33 | 89.1% |
| Manual Rifampin | Resistant | 4 | 10.9% |

**Discussion**

In our study; the sensitivity and specificity of ZN stain were 86.5% and 100% respectively which is in slightly higher than that reported by Rahman et al., 2015\(^{(8)}\) who showed that sensitivity and specificity of ZN stain were 77.7% and 91.4% respectively. And also higher than Salem et al., 2014\(^{(9)}\) who showed that sensitivity and specificity of ZN stain were 78.8% and 91.2% respectively. And far from Iqbal et al., 2009\(^{(10)}\) who reported that that sensitivity of ZN stain was 66.2%. This could be due to the fact that most of specimens of new cases received in our study came from patients suspected clinically and radiologically to have pulmonary tuberculosis. In our study, the recovery rate of L-J culture was 37 isolates out of 37 (100%). This finding is relatively higher than the range of earlier studies e.g. Somoskovi et al., 2000\(^{(11)}\) and Nair et al., 2009\(^{(12)}\) found that the recovery rate of LJ medium in their studies was, 80.7% and 61.9% respectively. Our results were also higher in comparison to the results of El-Bohy et al., 2009\(^{(13)}\) who found the recovery rate on LJ medium was 53% but only
smear-negative suspected TB cases were included in their study. In our study, positivity of GeneXpert in pulmonary cases was about 86.5% which is similar to that reported by Darwish et al., 2013\(^ {14}\) who showed positivity of GeneXpert was 82.3%. Also this study is in agreement with Buchelli et al., 2014\(^ {15}\) who showed positivity of 82%. However, Munir et al., 2015\(^ {16}\) reported a positivity of 77% while Tan et al., 2017\(^ {17}\) showed that positivity of GeneXpert was about 36.3%. These variations in results may be due to various factors, such as sample processing. Our finding of PPV of GeneXpert in pulmonary cases was 100% which is in agreement with some earlier studies\(^ {14, 16}\). In contrast, Tang et al (2017)\(^ {17}\) reported a PPV of 78.2%. The Negative Predictive value of GeneXpert in the current study was 80.0% which agrees with that reported by others\(^ {14, 17}\) However, higher values (94.6%) were previously reported\(^ {18}\). According to our results, Z-N smear was slightly less sensitive than GeneXpert MTB/RIF test 86.5% vs. 90%. This is because Z-N smear methods requires 5000 to 10000 bacilli/ml of specimen\(^ {39}\). The performance of GeneXpert MTB/RIF assay in all studied tuberculosis cases (pulmonary and extra pulmonary) was 90% sensitivity which is in agreement with previous studies\(^ {17, 19}\). However, higher sensitivities ranging from 97% to 100% was reported by others\(^ {14, 20-22}\).

### Table 4: Gene expert results of all Tuberculosis patients

| GeneXpert | Number | Percent |
|-----------|--------|---------|
| Positive  | 45     | 90.0%   |
| Negative  | 5      | 10.0%   |

### Table 5: The performance of Gene Expert in detection of all studied patients

| GeneXpert | Sensitivity | Specificity | PPV   | NPV   | Accuracy |
|-----------|-------------|-------------|-------|-------|----------|
| All cases | 90.0%       | 100.0%      | 100.0%| 80.0% | 92.8%    |
| Pulmonary | 86.5%       | 100.0%      | 100.0%| 80.0% | 91.2%    |
| Extra-pulmonary | 100.0%  | 100.0%      | 100.0%| 100.0%| 100.0% |

### Table 6: Rifampin sensitivity by GeneXpert in all TB cases

| Gene expert | Rifampin | All TB  |
|-------------|----------|--------|
|             | N=50     | No     | %     |
| Sensitive   | 45       | 90%    |
| Resistant   | 5        | 10%    |

Our finding of specificity all tuberculosis cases (pulmonary and extra pulmonary) was 100% which is in agreement with Darwish et al., 2013\(^ {14}\) and Munir et al., 2015\(^ {16}\) however, lower specificities ranging from 91-93% were by others\(^ {18, 23}\). Regarding Rifampin sensitivity in pulmonary cases we found that using GeneXpert was sensitive in 32 cases (86.4%) of patients and resistant in 5 cases (13.6%) while, Rifampin sensitivity by LJ medium was sensitive in 33 (89.1%) of cases and resistant in 4 cases (10.9%) and this is in agreement with other studies that reported rifampin sensitivity using GeneXpert in 94.4%\(^ {23}\), 95%\(^ {24}\) and 98.6%\(^ {25}\).
Conclusion

Our findings suggest GeneXpert as a sensitive and specific powerful diagnostic method in patients of suspected pulmonary tuberculosis either AFB smear negative or positive due to its rapidity and simultaneous detection of Rifampicin resistance while, Conventional methods for detection of MTBC in clinical specimens have low sensitivity compared to molecular techniques. Thus, GeneXpert system would change the field of TB with rapid diagnosis.

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