



Detection of *Mycobacterium tuberculosis* - Microscopy to Molecular Techniques at the Tertiary Care Hospital in Telangana, India

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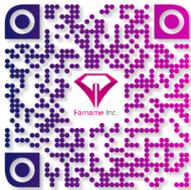
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Abstract

Background and Aims: Tuberculosis kills more than 1 million people every year, most of them in low-income and middle-income countries. An understanding of the trends in tuberculosis incidence, prevalence, and mortality is crucial to track the success of tuberculosis control programs. Microbiological diagnosis of diseases caused by Mycobacteria should be fast and effective to prevent contagions and optimize the management of infections.

Materials and Methods: A total of 1412 clinical pulmonary and extra pulmonary specimens were studied from January 2017 to December 2017 at Nizam's Institute of Medical Sciences, Hyderabad. All specimens were processed according to standard operating procedures. All the specimens were subjected to microscopy, culture, GeneXpert.

Results: Among 1412 samples received 813 were males (57.6%) and 599 females (42.4%). Among these 818 (57.9%) were pulmonary samples and 594 extra pulmonary samples. Mycobacterium prevalence was (21.6%) out of which Mycobacterium tuberculosis was found in 18.3% and Non tuberculous Mycobacteria (3.25%). The contamination rate was 2.6% (37 out of 1412). Among the positives, the most common affected age group was 21-30 yrs (22.2%). About 64 (4.53%) were smear positive. A total of 200 isolates (14.16%) were recovered by at least one culture LJ medium or BacT Alert 3D system. MTB was recovered in 216 (15.29%) by GeneXpert. MDRTB was detected in 8 (3.7%) by GeneXpert.

Conclusion: *Mycobacterium tuberculosis* complex is responsible for immense worldwide morbidity and mortality. Delays in diagnosis may postpone administration of appropriate treatment and be detrimental to patient outcomes. Since traditional culture methods are slow, newer molecular techniques allow more rapid and sensitive laboratory diagnosis of tuberculosis.

Keywords: Pulmonary, Extra pulmonary, Contamination, Smear, Culture, GeneXpert

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تشخیص مایکروباکتریوم به روش‌های مولکولی و کشت از بخش مراقبت‌های ویژه بیمارستان TELANGANA

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چکیده

اطلاعات مقاله

زمینه و اهداف: بیماری سل همه‌ساله بیشتر از یک میلیون نفر را در میان کشورهای با درآمد کم و یا متوسط می‌کشد. درک روند وقوع، شیوع و مرگ‌ومیر توپرکلوزیس (سل) برای پیگیری موفقیت در برنامه‌های کنترل آن بسیار مهم است. تشخیص میکروبیولوژیکی بیماری‌های ناشی از مایکوباکتری‌ها باید سریع و مؤثر باشد تا از ابتلا به بیماری جلوگیری و مدیریت عفونت را بهینه کند. لذا هدف مطالعه تشخیص مایکوباکتریوم توپرکلوزیس به روش مولکولی و کشت از بخش مراقبت‌های ویژه بود.

مواد و روش کار: در مجموع، ۱۴۱۲ نمونه بالینی سل ریوی و خارج ریوی از ژانویه تا دسامبر ۲۰۱۷ در انستیتوی علوم پزشکی نظام، حیدرآباد، پاکستان، مطالعه شد. تمام نمونه‌ها مطابق رویه‌های عملیاتی استاندارد پردازش شدند. تمام نمونه‌ها با میکروسکوپ، کشت، و با کمک دستگاه تشخیص مولکولی GeneXpert بررسی شدند.

یافته‌ها: از ۱۴۱۲ نمونه دریافت‌شده ۸۱۳ نفر مرد (۵۷/۶٪) و ۵۹۹ زن (۴۲/۴٪) بودند. از بین این نمونه‌ها، ۸۱۸ مورد (۵۷/۹٪) نمونه سل ریوی و ۵۹۴ نمونه خارج ریوی وجود داشت. شیوع مایکوباکتریوم ۲۱/۶٪ بود که از این تعداد مایکوباکتریوم توپرکلوزیس در ۱۸/۳٪ و مایکوباکتریوم‌های غیر توپرکلوزیس (۳/۲۵٪) مشاهده شد. میزان آلودگی ۲/۲۶٪ (۳۷ مورد از ۱۴۱۲) بود. در میان موارد مثبت، شایع‌ترین گروه سنی مبتلا ۳۰-۲۱ سال (۲۲/۲٪) بود. حدود ۶۴ نفر (۴۲/۴ درصد) اسمیر مثبت داشتند. در مجموع ۲۰۰ ایزوله (۱۴/۱۶٪) با حداقل یک سیستم کشت L₁ یا سیستم BacT Alert 3D با زبایی شدند. در MTB در ۲۱۶ (۱۵/۲۹) توسط GeneXpert با زبایی شد. MDRTB در ۸ (۳/۷٪) توسط GeneXpert تشخیص داده شد.

نتیجه‌گیری: کمپلکس مایکوباکتریوم توپرکلوزیس مسئول عوارض و مرگ و میر عظیمی در سراسر جهان است. تاخیر در تشخیص ممکن است استفاده از درمان مناسب را به تعویق اندازد و برای بیمار مضر باشد. به دلیل طولانی بودن زمان کشت سنتی، تکنیک‌های جدید مولکولی امکان تشخیص سریع آزمایشگاهی و حساس‌تر توپرکلوزیس را فراهم می‌کند.

کلید واژه‌ها: ریوی، خارج ریوی، آلودگی، اسمیر، کاشت، GeneXpert

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کپی‌رایت © مجله میکروبیولوژی پزشکی ایران: دسترسی آزاد؛ کپی‌برداری، توزیع و نشر برای استفاده غیرتجاری با ذکر منبع آزاد است.

Introduction

Every year tuberculosis kills more than 1 million people from low-income and middle-income countries (1-3). Thorough understanding of the trends in tuberculosis incidence, prevalence, and mortality helps to identify intervention challenges for tuberculosis care and prevention and in successful implementation of tuberculosis control programs. However, evaluation of these trends is challenging (1-3). Currently the global strategy to control TB is through preventing infection by efficient case finding and treatment and which helps to stop the infection from progressing to an active disease (4).

Worldwide, 9.6 million people were estimated to have TB in 2014 (4). Of all the countries that report their TB statistics to WHO, there are 22 countries, including India, that are referred to as the TB "high burden" countries; and they have been prioritized at a global level since 2000. They accounted for 82% of all estimated cases of TB world-wide in 2014 (5). According to the WHO statistics for 2014, the estimated incidence of TB in India is 2.2 million cases which accounts for approximately 1/5th of global incidence (6). In addition at least 2.7 lakh (270,000) Indians die of TB every year (7). It is estimated that about 40% of the Indian population is infected with TB with an estimate of approximately 794,046 sputum smear-positive cases reporting to RNTCP in 2014 and an overall treatment

success rate for new TB patients and re-treatment patients success rate being 88% and 70% respectively (7).

Microbiological diagnosis of diseases caused by Mycobacteria should be fast and effective to prevent contagions and optimize the management of infections and is essential to provide appropriate antimicrobial therapy, in the case of *Mycobacterium tuberculosis* (*Mtb*), and to implement effective infection control or public health interventions (8,9).

The culture represents a decisive step for the diagnosis, treatment and control of TB, but the labor in culture on solid media is intensive and it may take several weeks for colonies to become detectable; even then, the process may require further subculture for definitive identification. The combination of solid and liquid media is currently regarded as the “gold standard” for primary isolation of Mycobacteria in clinical samples, and turnaround times not exceeding 21 to 30 days after specimen collection (10).

During last decades there have developed automated systems for detection of growth in different microorganisms in liquid medium. Most automated systems are based on different technologies, such as colorimetric methods that detect bacterial CO₂ production like BacT/ALERT 3D system, radiometric detection methods, such as Radiometric BACTEC 460 System, others use pressure sensors or fluorometric methods to detect bacterial O₂ consumption, such as the ESP Culture System II and BACTEC MGIT 960 System, respectively. A considerable number of these methods provide similar times to detection, with fully automated instruments or without the need for any instrumentation (11,12).

The introduction of amplification techniques in the mycobacteriology laboratory is going to provide faster and more accurate detection of *Mtb* complex from respiratory and extra-pulmonary specimens (10). The rapid detection of *M. tuberculosis* and rifampin (RIF) resistance in infected patients is essential for disease management, because of the high risk of transmission from person to person and emergence of MDR-TB and extensively drug resistant (XDR) tuberculosis. Culture is the “gold standard” for final determination, but it is slow and may take up to 2 to 8 weeks. Although smear microscopy for acid-fast bacilli (AFB) is rapid and inexpensive, it has poor sensitivity and a poor positive predictive value (PPV). Thus, rapid identification, which is essential for earlier treatment initiation, improved patient outcomes, and more effective public health interventions, relies on nucleic acid amplification techniques (13).

The GeneXpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and hemi-nested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens (14,15). 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 h (14,15).

Materials and Methods

A total of 1412 clinical pulmonary and extra pulmonary specimens were studied from January 2017 to December 2017 at Nizam's Institute of Medical Sciences, Hyderabad. All specimens were processed according to standard operating procedures. All the specimens were subjected to microscopy, culture and GeneXpert.

Blood and the specimens that were inoculated by one culture method were excluded from this study. The specimens studied were inoculated in parallel in Löwenstein Jensen (LJ) solid medium and liquid medium (automated system BacT/ALERT MB BacT 3D system).

Samples Processing

Inoculation on LJ Medium “Gold Standard”

Pulmonary and extra-pulmonary (except sterile body fluids) specimens were liquefied and decontaminated with modified Petroff's method to eliminate the normal flora/contaminants using 4% NaOH. After processing the samples, 0.1 mL was inoculated in LJ medium and incubated at 37°C for 8 weeks. Specimens collected from sterile sites were concentrated by centrifugation (3000 g for 15 minutes) without prior decontamination. Then 0.1 ml were inoculated in LJ medium and incubated at 37°C for 8 weeks. The readings of cultures were done weekly for 8 weeks. The final identification was done by conventional biochemical tests (niacin and catalase etc.), according to the standard procedures (16).

Inoculation in MP Bottle for BacT/ALERT 3D

The MP bottle has 10 mL of liquid medium (7H9 Middlebrook) with casein, bovine serum albumin and catalase and 0.5 mL of antibiotic supplement MB/BacT (amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin) to reduce other bacterial contamination. Then, 0.5 mL of the digested and decontaminated sample was inoculated in a bottle and incubated in the BacT/ALERT 3D system.

Sterile body fluids were inoculated in MP bottle with 0.5 mL of reconstitution fluid and 0.5 mL of samples which were previously centrifuged at 3000g for 15 minutes.

After sample inoculation, the MP bottles were loaded in the BacT/ALERT 3D instrument and incubated for 6 weeks. The BacT/Alert system is a self-contained incubator, shaker, and detector. Each well contains a colorimetric detector. The instrument scans each well once every 10 min. After amplification and filtering, voltage signals are digitized and transmitted to a microcomputer for analysis. BacT/Alert tests for CO₂ production in each bottle 144 times per day. The data points are plotted as reflectance units versus time and result in a growth curve. The algorithm for detection of growth is based on an analysis of the rate of change of CO₂ concentration in each bottle. It then alerts the operator for the presence and location of positive bottles.

All samples that were identified as positive by the instrument BacT/ALERT 3D were subjected to ZN staining. If ZN staining confirmed AFB, the result was considered positive (true positive). If staining did not reveal AFB, 0.2 mL was transferred to LJ medium (subculture) and re-incubated at 37°C in an incubator (not in the instrument) for 4 weeks. If the growth from LJ was confirmed as AFB positive by ZN staining then it was considered as true positive by the instrument.

Any sample initially flagged positive by the instrument but smear negative and no mycobacterial growth detected on LJ subculture, was considered as negative (false positive by the instrument).

GeneXpert testing was performed according to the manufacturer’s instructions. Sample reagent was added to untreated sample at a ratio of 2:1, manually agitated and kept for 10 min at room temperature, then shaken again and kept for 5 min; 2 ml of the inactivated material was transferred to the test cartridge and inserted into the test platform.

The bottles which flagged positive by the instrument and confirmed the presence of AFB by ZN stain were subjected to MPT 64 for differentiation of MTB complex from non-tuberculous bacteria for which an extract of 100 µL was used for immunochromatographic assay BIOLINE SD (Standard Diagnostics, Kyonggi-do, Korea) for the identification of MPT64 antigen, present only in *Mtb*

complex. The interpretation of SD BIOLINE test results was performed according to the manufacturer’s instructions.

Statistical Analysis

Statistical analysis of data was performed using the statistical programs Microsoft excel and SPSS 25 (SPSS Inc., Chicago, Illinois, USA).

Results

A total of 1412 samples received from January 2017 to December 2017 were processed. Of which 813 were males (57.6%) and 599 females (42.4%). About 873 samples were from inpatients (61.8%) and 539 from out patients (38.17%). Among these, 818 (57.9%) were pulmonary samples and 594 extra pulmonary samples (42.1%) (Tables 1,2).

Mycobacterial culture was detected in 305 cases (21.6%) out of which Mycobacterium tuberculosis was found in 259 (18.3%) and Non tuberculous Mycobacteria 3.25%. The contamination rate was 2.6% (37 out of 1412). Among the positives, the most common affected age group was 21-30 yrs (22.2%) followed by 41-50 and 51-60 (17.3%). Among the pulmonary samples bronchial wash showed more positivity (28.5%) and among extra pulmonary samples pus and FNAC samples showed high positivity (45, 50%) (Tables 1,2).

Table1. Distribution of pulmonary samples

S.No.	Sample	Number	Positives
1	Sputum	153(18.7%)	35(22.8)
2	Bronchial wash	665(81.2%)	190(28.5%)
Total		818	225 (27.5%)

Table2. Distribution of extra-pulmonary samples

S.No.	Sample	Number	Positives
1	Tracheal aspirate (Fluid)	2(0.3%)	0
2	Ascitic fluid	53(8.9%)	3(5.6%)
3	Ryles tube aspiration	1(0.16)	0
4	Pleural fluid	97(16.3)	8(8.24%)
5	CSF	213(35.8)	19(8.9%)
6	Pus	44(7.4%)	20(45.45%)
7	Synovial fluid	39(6.6%)	1(2.56%)
8	Lymph node	34(5.7%)	12(35.3%)
9	FNAC	6(1.01)	3(50%)
10	Peritoneal	7(1.17)	0
11	Tissue	59(9.93)	11(18.6%)

S.No.	Sample	Number	Positives
12	Aspirate	12(2%)	2(16.6%)
13	Pericardial	5(0.84%)	0
14	CAPD	3(0.5%)	0
15	Gastric aspirate	6(1)	0
16	Lung biopsy	1(0.16)	0
17	Bone marrow aspirate	4(0.67)	0
18	Drain fluid	2(0.3)	0
19	Lymph node biopsy	1(0.16)	0
	Total	594	79 (13.29%)

Microscopy

About 64 (4.53%) were smear positive by auramine-rhodamine stain. Out of which 56 were pulmonary (3.96%) and 8 extra-pulmonary (0.56%).

A total of 200 isolates (14.16%) were recovered by at least one culture i.e. LJ medium or BacT Alert 3D system. Of these 154 (77%) were identified as Mtb, and 46 isolates (23%) as non-tuberculous mycobacteria. Of the 200, 166

(83%) were detected on LJ medium and 189 (94.5%) in the BacT Alert 3D system. On the other hand, 11 (5.5%) were detected only on LJ medium and 34 (17%) only in the BacT Alert 3D system. (Table 4).

There was good concordance in the culture results between both culture methods, with an agreement rate of 94.8% (kappa coefficient, 0.884; 95% confidence interval (CI), 0.8269–0.9413

Table 3. Microscopy versus culture positives

Smear	Culture +ve	Culture negative
Smear positive	45(tp)	19 (fp)
Smear negative	155(fn)	1193(tn)

Sensitivity - $45/45+155=22.5\%$, specificity - $1193/1193+19=98.4\%$, positive predictive value - 70.3%, negative predictive value - 88.5%, likelihood ratio - 14

Table 4. BacT Alert versus LJ

Culture	Lj+ve	Lj-ve
BacT alert +ve	155	34
BacT alert -ve	11	1216

Sensitivity - $155/155+11=93.37\%$, specificity - $1216/1216+34=97.2\%$, ppv - 82%, npv - 99%, likelihood ratio - 33.3

MTB was recovered in 216 (15.29%) by GeneXpert. Both culture and GeneXpert were positive in 111 and only GeneXpert in 105.

Extra-pulmonary samples - A total of 494 extra-pulmonary samples were received out of which maximum number was from body fluids (415, 84%), high positivity

was reported from pus samples (39.1%) followed by tissue (23.7%) (Table 5). GeneXpert showed sensitivity from 48.1% to 79.16% and specificity of 90% to 92% (Table 6) and among the various diagnostic methods BacT Alert showed high sensitivity and specificity (Table 7).

Table 5. Comparison of positives among extra pulmonary samples by various methods

Type of extra-pulmonary sample	Total number of samples	Total number of positives by any method	GeneXpert positives	Culture positives
Body fluids	415	30(7.2%)	24(5.78%)	15(3.6%)
Aspirates	33	5(15.15%)	5 (15.15%)	0
Pus	46	18(39.1%)	15(32.6%)	12(26.08%)
Tissue	97	23(23.7%)	10(10.3%)	7(7.2%)
CAPD	3	0	0	0

Table 6. GeneXpert with culture as reference

S.No.	Sample	Sensitivity	Specificity	+ve predictive	-ve predictive
1.	Sputum	79.16%	91.4%	63.3	95.9
2.	BAL	48.1%	89.6%	54.16	87.15
3.	Extrapulmonary	65.8%	92.94	40.9	97.3
4.	Overall	55	91.3%	51.38	92.5%

Table 7. Comparison of various tests with culture

Type of test	Sensitivity	Specificity	ppv	Npv	Likelihood ratio
Microscopy	22.5	98.4	70.3	88.5	14
Bact alert culture	93.37	97.2	82	99	33.3
GeneXpert	55.5	91.3	51.38	92.5	2

MDRTB was detected in 8 (3.7%) by GeneXpert.

Discussion

Early diagnosis of tuberculosis is essential for initiating an effective treatment regimen and preventing its transmission in the community (17).

In our study only 0.56% were smear positive. Culture was positive in 41 (6.9%) and GeneXpert in 11.11%.

AFB microscopy is cheap, except for the microscope, requires little material and gives results in a day. Since 1975 individual studies reported a great disparity in the sensitivity values, from 33% to 80% (18,19). The most recent global report indicates that even among PTB cases the sensitivity of this method is only 40–58%. Although AFB microscopy is believed to be very specific, however, the differential identification of mycobacterium species by morphological parameters is almost impossible (20).

In Ali Nour *et al.* study, sensitivity and specificity of AFB microscopy in poor area lab and in reference lab were 29.6%, 81.8% and 86.1%, 99.4%, respectively (21). In our study the sensitivity and specificity of smear microscopy was 22%, 98.4% which was similar to study conducted by Monika Agarwal while it was 54.3% and 99.6%, respectively in Sang Hee Park *et al.* study (22,23).

The contamination rate obtained for BacT/ALERT 3D (2.6%) was within in the international parameters (4%-7%); while Maria *et al.* reported 4.6% (24).

The sensitivity, specificity obtained for BacT/ALERT 3D instrument was acceptable for a good performance of this method (93.4%, 97.2% respectively). It has been documented in international literature that the sensitivity values for the MB/BacT/ALERT 3D System may be between values of 78% and 99%. In Mari *et al.* study, it was 89.9%, which was superior to that obtained by Sorlozano *et al.* (24).

Numbers of studies have demonstrated the utility of GeneXpert in diagnosis of pulmonary tuberculosis. In our study, overall sensitivity, specificity, PPV and NPV of GeneXpert were 55%, 91.3%, 51.38%, 92.5%, while it

was 86.8%, 93.1%, 78.5% and 96% respectively in the study conducted by Monika Agarwal *et al.* while Lawn *et al.* reported the sensitivity and specificity of Xpert MTB/RIF were 79.0% and 97.3%, respectively which was comparable with other studies (25-30).

In the other studies, GeneXpert sensitivity and specificity for BAL sample was from 81%-92% and 71%-100% (25-29); while it was 48.1% and 89.6% in our study.

Among the extra-pulmonary samples, maximum received samples were CSF, 213(35.8%) followed by tissue 59 (9.3%). High positivity was obtained from pus and tissue samples (45%, 39.5%) while Lawn and Zumla *et al.* reported high positivity from aspirates (35%) and gastric aspirates (23%) (30).

The MDRTB prevalence by GeneXpert was 3.7% in our study while it was 9.2% in study conducted by Raghu Prakash Reddy *et al.* (31).

Conclusion

M. tuberculosis complex is responsible for immense world-wide morbidity and mortality. Delays in diagnosis may postpone administration of appropriate treatment and be detrimental to patient outcomes. As a slow-growing organism using traditional culture methods, newer molecular techniques allow for more rapid and sensitive laboratory diagnosis of tuberculosis. NAA tests to provide early indication of drug resistance.

In conclusion, specificity of GeneXpert is high in majority of EPTB cases. The sensitivity was low for BAL samples. Maximum positivity was seen with pus samples. These findings support recent WHO guidelines regarding the use of GeneXpert for TB diagnosis from EPTB specimens.

BacT/ALERT 3D system is a suitable method for recovering tuberculous and non-tuberculous mycobacteria from clinical samples. With a shorter time to detection providing a faster initiation of treatment, especially those

with AFB smear negative specimens. It was proven to be more efficient than LJ medium in isolation of mycobacteria. In addition, the application of PCR assay directly on positive liquid media of automated systems allows confirmation of the results and fast identification of *M. tuberculosis* which was very useful to provide faster treatment and a better prognosis in patients with AFB smear negative.

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Conflict of interest

The authors declared that there is no conflict of interest to declare.

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