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Original Article

Microbiology Section

Diagnostic Performance and Comparative Analysis of Conventional Drug Susceptibility Testing, Line Probe Assay, and GeneXpert in Extrapulmonary Tuberculosis Cases: A Cross-sectional Observational Study

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ABSTRACT

Introduction: Tuberculosis (TB), a known threat to mankind for ages, causes a significant burden on healthcare worldwide. The alarming rise of Extrapulmonary Tuberculosis (EPTB) has led healthcare professionals to opt for molecular diagnostics. Despite the availability of rapid diagnostics, conventional culture Drug Susceptibility Testing (DST) is still considered the gold standard in diagnosing TB.

Aim: To assess the diagnostic accuracy of culture DST which is considered the gold standard.

Materials and Methods: This cross-sectional observational study included 150 suspected EPTB patients and was conducted over a period of one year (September 2018 to August 2019) at Lokmanya Tilak Municipal Medical College and Sion Hospital (LTMMC), a tertiary care hospital in central Mumbai, Maharashtra, India. The hospital is located adjacent to Dharavi, the largest slum area with a high population density, which contributes to the majority of patients attending clinics. Patients attending the pulmonary medicine Outpatient Department (OPD) with extrapulmonary manifestations and suspected cases of EPTB were enrolled in the study. Samples, excluding sputum, were sent to the Department of Microbiology, LTMMC, Sion Hospital for GeneXpert analysis. The samples were further evaluated by microscopy, GeneXpert, and culture DST to detect the presence of *Mycobacterium tuberculosis (M. tuberculosis)* and drug susceptibility, respectively. Patients positive for Pulmonary Tuberculosis (PTB) were excluded from the study. Categorical variables were described using percentages. A Chi-square test was applied, and a p-value of <0.05 was considered significant. All statistical analyses were performed using SPSS statistical software (IBM SPSS version 26.0, Armonk, N.Y.).

Results: Out of the 150 extrapulmonary samples, 23 (15.33%) samples were culture positive for *M. tuberculosis* and were subjected to DST using the 1% proportion method and GeneXpert assay. The MDR isolates were tested using the first-line Line Probe Assay (LPA). Eleven (47.8%) showed resistance to first-line antitubercular drugs. Among the 12 new cases, only 2 (16.7%) showed resistance compared to 9 (81.8%) in previously treated cases. A discordance of 8.7% was observed between DST with GeneXpert and LPA. Additionally, a discordance of 8.7% was observed between DST and LPA for rifampicin resistance and 4.3% for isoniazid resistance.

Conclusion: The paucibacillary nature of extrapulmonary samples contributes to the challenging diagnosis of EPTB cases, leading to increased drug resistance. Highlighting the importance of the conventional solid culture DST method, this study strongly recommends the use of conventional DST accompanied by LPA for extrapulmonary cases.

Keywords: Antitubercular drugs, Diagnosis, Drug resistance

INTRODUCTION

Tuberculosis (TB) is a communicable disease caused by the *M. tuberculosis* complex. Initially affecting the lungs, TB progresses to pulmonary TB (PTB) and extrapulmonary TB (EPTB), characterised by infection in other body organs [1]. The infection commonly spreads through small droplet nuclei shed when an infected person coughs or sneezes. EPTB affects organs other than the lungs, with the exception of miliary TB. Diagnosis of EPTB is challenging due to its atypical presentations, often leading to misdiagnosis. It constitutes around 15-20% of all TB cases [2]. Conventional diagnostic methods struggle to diagnose EPTB due to the lengthy testing time, sampling difficulty, and the paucibacillary nature of samples [3]. Limited information is available regarding drug resistance in EPTB, especially in high-burden countries like India [4], primarily due to the difficulty in obtaining diagnostic specimens and the limited number of national laboratories equipped to conduct culture and DST for *M. tuberculosis* from extrapulmonary specimens. Clinical symptoms are the main prognostic indicators in EPTB, and suspicion of drug resistance arises only after failure or non response to first-line therapy. Since culture DST evaluation for extrapulmonary samples is rarely presented in published literature, only the advantage of upfront DST by Gene Xpert [5] is highlighted. Therefore, understanding the methodology and purpose of conventional culture DST in the era of molecular diagnostics is crucial. With the rise of drug resistance in TB patients, culture DST holds great value, as the presence of gene mutations may lead to false-negative results in molecular tests like LPA [6] and Gene Xpert. This study aims to assess the diagnostic accuracy of culture DST as the gold standard and to evaluate its performance in comparison with LPA and Gene Xpert for the diagnosis of EPTB. The study addresses the burden of drug resistance in EPTB.

MATERIALS AND METHODS

This cross-sectional observational study, involving 150 suspected EPTB patients, was conducted over one year (September 2018 to August 2019) at Lokmanya Tilak Municipal Medical College and Sion Hospital, a tertiary care hospital in central Mumbai, Maharashtra, India. The study protocol received approval from the Institutional Ethics Committee (IEC) (IEC/LTMMC761/19) with registration number ECR/266/Lokmanya/Inst/MH/2013RR-16. Informed consent was obtained from all enrolled patients, and consent from guardians was secured for patients under 18 years of age.

Inclusion criteria: Patients visiting the outpatient chest and TB department at the centre, as well as those admitted with suspicion of EPTB were included in the study.

Exclusion criteria: Patients who were tested positive for PTB were excluded from the study.

Sample size calculation: The sample size, calculated using the formula for an infinite population, (where the population is greater than 50,000) = $Z^2 \times (p) \times (1 - p) C^2$ considering a confidence interval of 95% and a margin of error of 6%, and a prevalence percentage of 15-20% for an unlimited population size, was determined to be 144. Rounding off resulted in a sample size of 150 [7].

Study Procedure

A total of 150 extrapulmonary samples, such as pus, urine, Cerebrospinal Fluid (CSF) and tissue biopsy, were received and tested in the Department of Microbiology. Standard microbiological diagnostics, including acid-fast staining microscopy, culture using Lowenstein-Jensen media, and molecular techniques like GeneXpert® Cartridge based Nucleic Acid Amplification Test (CBNAAT) manufactured by Cepheid Diagnostics, were employed. Drug resistance testing for isoniazid and rifampicin was performed using the 1st line LPA (GenoType® *M. tuberculosis* DRplus VER 2.0). The samples, except for sterile body fluids, were decontaminated using the 2% N-acetyl-l-cysteine–sodium hydroxide (NALC-NaOH) method and cultured on Lowenstein Jensen (LJ) medium. The 23 positive cultures were confirmed using the SD BIOLINE MPT-64 kit [8] for *M. tuberculosis* and then subjected to 1st line DST using the 1% proportion method, GeneXpert assay, and LPA (1st line), respectively. Ziehl-Neelsen staining [9] was performed for all the samples.

(a) **Proportion method of Drug Susceptibility Testing (DST):** On LJ medium with drug concentrations as mentioned in [Table/Fig-1], susceptibility testing of culture-positive specimens was conducted. Colonies were removed from the LJ slant and homogenised using glass beads and a vortex mixer in sterile distilled water. The turbidity of each suspension was adjusted to meet the McFarland No. 1 turbidity criterion. Two homogenised bacterial suspension dilutions, 10^3 and 10^5, were inoculated on each of the drug-containing media. Additionally, both suspensions were inoculated on a drug-free medium as a positive control. Any isolate showing more than 1% growth on the medium containing the drug, compared to the control, was labeled as a resistant strain. The standard sensitive strain H37Rv was used as a control strain [10].

Drug media	Concentration (µg/mL)	Critical proportion to determine the resistance (%)
Isoniazid	0.2	1
Rifampicin	40	1
Ethambutol	2	1
Streptomycin	4	1

[Table/Fig-1]: Drug concentrations used in 1% proportion method of DST.

(b) GeneXpert assay was performed on all considered samples at the centre. The GeneXpert assay for *M. tuberculosis* works based on the principle of the CBNAAT [11].

(c) Furthermore, the 23 culture-positive isolates underwent 1st line LPA at a referral centre in our city, and the results were obtained. LPAs are a family of DNA strip-based tests that allow users to determine the drug resistance profile of an *M. tuberculosis* complex strain by interpreting a pattern of bands representing lines of immobilised probes bound (or hybridised) to *M. tuberculosis* complex amplicons (DNA amplification products) [12].

Important definitions include [13]

- Mono resistance: Resistance to only one first-line antitubercular drug.
- Multidrug Resistance (MDR): Resistance to atleast both isoniazid and rifampicin.
- Polyresistance: Resistance to more than one first-line antitubercular drug, excluding both isoniazid and rifampicin.

STATISTICAL ANALYSIS

Categorical variables were described using percentages. A Chi-square test was applied, and a p-value of <0.05 was considered significant. All statistical analyses were performed using SPSS statistical software (IBM SPSS version 26.0, Armonk, N.Y.).

RESULTS

Out of 150 patients, 25 (16.7%) were culture-positive for tuberculosis. Among these, 23 were positive for *M. tuberculosis* and 2 were identified as Non Tuberculous Mycobacteria (NTM). The NTM isolates were not further processed, and susceptibility testing was only conducted for the *M. tuberculosis* isolates.

Patterns of resistance to first-line anti-tubercular drugs among patients: Out of the 150 extrapulmonary samples, 23 (15.33%) were culture-positive for *M. tuberculosis* and subjected to culture drug susceptibility testing (DST). Among the *M. tuberculosis* positive patients, 11 (47.8%) showed resistance to first-line antitubercular drugs [Table/Fig-2].

Resistance		No. of patients	Percentage
Mono resistance	INH only	00	-
	RIF only	03	13.0
	EMB only	00	-
	SM only	02	8.7
MDR	INH+RIF	05	21.7
	INH+RIF+EMB	00	-
	INH+RIF+SM	00	-
	INH+RIF+EMB+SM	00	-
Polyresistance*	INH+EMB+SM	01	4.3
	INH+SM	00	-
	RIF+SM	00	-
	EMB+SM	00	-
Total resistance		11	47.8

[Table/Fig-2]: Patterns of resistance of *M. tuberculosis* isolates to the first-line antitubercular drugs identified by 1% proportion method: (n=23).
*Resistant to two or more drugs but not both isoniazid and rifampicin; EMB: Ethambutol; INH: Isoniazid; MDRTB: Multidrug-resistant TB (resistant to at least both isoniazid and rifampicin with or without resistance to other drugs); RIF: Rifampicin; SM: Streptomycin

Distribution of *M. tuberculosis* susceptibility based on treatment history: Among 12 new cases, only 2 (8.7%) showed resistance to Streptomycin, while 9 (39.1%) previously treated cases showed resistance to Rifampicin (3), Isoniazid and Rifampicin (5), and Isoniazid, Ethambutol, and Streptomycin (1) [Table/Fig-3].

Comparing the results of conventional DST with Gene Xpert assay and LPA for Rifampicin susceptibility: Among the 23 isolates, DST showed eight with rifampicin resistance, while both Gene Xpert and LPA identified 10 with rifampicin resistance, resulting in an 8.7% discordance between DST and both Gene Xpert and LPA [Table/Fig-4].

Case	Susceptible	Resistant	Total
New case	10 (43.5%)	02 (8.7%)	12 (52.2%)
Previously treated	02 (8.7%)	09 (39.1%)	11 (47.8%)
Total	12 (52.2%)	11 (47.8%)	23

[Table/Fig-3]: Treatment history in *M. tuberculosis* culture-positive cases (n=23). p-value<0.05 significant (Chi-square=7.326, p-value=0.0067)

Drug tested	DST	Gene Xpert	LPA	Discordance (%)
Rifampicin resistant	08 (34.8%)	10 (43.5%)	10 (43.5%)	2 (8.7%)
Rifampicin susceptible	15 (65.2%)	13 (56.5%)	13 (56.5%)	
Total	23	23	23	23

[Table/Fig-4]: Comparative results of DST with GeneXpert and Line Probe Assay (LPA) for Rifampicin resistance: (n=23). p-value>0.05 insignificant (Chi-square=0.4808, p-value=0.7863)

Comparing the results of conventional DST with LPA for mono or MDR analysis: DST showed eight isolates with rifampicin resistance and five with isoniazid resistance, while LPA identified 10 with rifampicin resistance and four with isoniazid resistance, resulting in a discordance of 8.7% for rifampicin resistance and 4.3% for isoniazid resistance [Table/Fig-5].

Susceptibility testing	DST by 1% proportion method	Line probe assay	Discordance
Susceptible to Rifampicin	15 (65.2%)	13 (56.5%)	02 (8.7%)
Resistance to Rifampicin	08 (34.8%)	10 (43.5%)	
Susceptible to Isoniazid	18 (78.3%)	19 (82.6%)	01 (4.3%)
Resistance to Isoniazid	05 (21.7%)	04 (17.4%)	

[Table/Fig-5]: Comparing results of drug susceptibility of 1st line Anti-Tb drugs by conventional DST, and Line Probe Assay (LPA): (n=23).

Comparing the smear and culture positivity in extrapulmonary samples: A comparison between primary smear and culture results of *M. tuberculosis* among patients showed a sensitivity of 48% and specificity of 96% for Ziehl-Neelsen (ZN) staining [Table/Fig-6].

ZN stain	Culture		Total
	Culture Positive	Culture Negative	
AFB seen	12 (48%)	05 (4%)	17 (11.3)
AFB not seen	13 (52%)	120 (96%)	133 (88.7%)
Total	25 (16.7%)	125 (83.3%)	150

[Table/Fig-6]: Comparison of Primary ZN smear with culture positive isolates (n=150).

DISCUSSION

India is a major contributor to the global burden of TB, with cities like Mumbai facing challenges due to high population density, low living standards, and poor sanitation, which contribute to the increasing number of extrapulmonary cases [14]. The sensitivity of Ziehl-Neelsen (ZN) staining was observed to be 48%, with a specificity of 96%. In a study by Ozkutuk N and Surucüoglu S the sensitivity and specificity of microscopic examination for extrapulmonary specimens were reported as 47.8% and 99%, respectively [15]. The variation in sensitivity could be attributed to the paucibacillary nature of extrapulmonary specimens and nonuniform distribution of bacilli within them. Furthermore, the higher smear positivity compared to culture positivity in present study may be attributed to interobserver variations in reporting microscopy findings. In present study, 23 (15.33%) samples were culture-positive for *M. tuberculosis*, and among them, 11 (47.8%) showed resistance to first-line antitubercular drugs. This culture positivity rate was consistent with the findings of a study by Dusthackeer A et al., which reported a culture positivity rate of 15.5% for extrapulmonary samples [14].

The majority of previously treated cases in present study showed acquired drug resistance, aligning with the research conducted by Maurya AK et al., in northern India, where patients with EPTB had an overall MDR TB rate of 13.4% [16]. In present study, MDR-TB was demonstrated in 21.7% of cases, indicating a potential sampling bias due to a majority of the samples being obtained from chronic TB sufferers. This finding contrasts with the study by Goyal V et al., which inferred an MDR-TB rate of 39.9% in Western states of India [17]. A discordance of two isolates (8.7%) was observed between DST, Gene Xpert, and LPA. These results, while statistically insignificant, are likely due to the small sample size and may not be applicable for a larger population. Similarly, in a study by Vadwai V et al., the Gene Xpert test correctly identified 98% of phenotypic rifampicin-resistant cases and 94% of phenotypic rifampicin-susceptible cases [18]. A discrepancy of six samples was observed between Gene Xpert and DST results. A 100% concordance was observed between Gene Xpert and LPA regarding the detection of rifampicin monoresistance. However, in a study by Yadav RN et al., the sensitivity of the Xpert *M. tuberculosis*/RIF assay and LPA were found to be 96% and 99%, respectively [19], indicating potential variations based on sampling regions, sampling approaches, and rpoB gene mutations among populations.

In the current study, only one sample was identified as isoniazid-resistant by conventional DST but was detected as susceptible by LPA. LPA (*M. tuberculosis*DRplus) detects low-level isoniazid resistance, including Inh A and katG probes for determining high-level isoniazid resistance. The discordant result might be explained by mutations, such as oxyR-ahpC and kasA genes, which are not detected by the LPA. Additionally, the detection limit of LPA is 10,000 colony-forming units per milliliter (CFU/mL), whereas culture has a detection limit of 10-100 CFU/mL [20,21]. Similarly, a study by Saglik I et al., noted lower sensitivity for the detection of isoniazid resistance with the *M. tuberculosis*DR assay [22].

Limitation(s)

The study had several major limitations. Firstly, the sample size was not large enough to cover the variety of strains exhibiting mutations. Secondly, the results of specificity and sensitivity of tests like LPA, DST, and Gene Xpert cannot be directly applied to a larger population. Despite these limitations, this study holds great relevance for readers. Limited data is available on drug resistance detection in EPTB compared to pulmonary TB in the Indian context. The study highlights the importance of targeting treatment based on the resistance profiles of patients, rather than blindly prescribing medication due to the lack of DST facilities in rural areas. This approach may contribute to tackling the increasing burden of drug-resistant TB. The results may not be extrapolated to a larger population which may be considered one of the limitations of this study.

CONCLUSION(S)

Although DST by the conventional culture method has the disadvantage of taking several weeks (28 days for culture and 42 days for DST), its importance cannot be ignored. This is especially true when Gene Xpert shows false-positive results due to patients being on treatment, experiencing relapse, or undergoing treatment after being lost to follow-up. Screening patients on a routine basis using Gene Xpert in such cases can misguide clinicians about the patient's response to treatment. Culture holds importance in cases where LPA fails to detect mutations other than rpoB, InhA, and Kat G. Therefore, the present study asserts that the conventional DST method remains the gold standard for diagnosing EPTB.

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