CONVENTIONAL DIAGNOSIS FOR TUBERCULOSIS VERSUS LATEST MODALITIES AT A TERTIARY CARE SETTING OF PESHAWAR

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ABSTRACT

Objective: To evaluate and compare the performance of Ziehl Neelsen (ZN) smearing, Lowenstein–Jensen (LJ) culture and real-time PCR analysis to detect Mycobacterium tuberculosis in pulmonary and extra-pulmonary specimens.

Materials and Methods: A descriptive study conducted from January 2016 through December 2018, on a total of 293 clinical samples of patients suspected with pulmonary and extra-pulmonary tuberculosis at a tertiary hospital of Peshawar, Pakistan. Specimens were processed by ZN smearing, LJ culture and Real-time PCR technique for the detection of M.tuberculosis. The target for the amplification was a segment of IS6110 in the M.tuberculosis chromosome. Specificity and sensitivity were derived for each test and Fishers exact test was performed to examine significance of association between specimen types and each test.

Results: Of the 293 samples, 165(56.3%) were from males and 128(43.7%) females. Mean age was 44 years (2-85 years). Specimen types included: CSF (30.4%), pleural fluid (4.1%), sputum (15%), urine (2.4%), synovial fluid (2.4%), other fluids (33.1%) and biopsies (12.6%). Using PCR as gold standard, ZN microscopy correctly identified 20.5% of total M.TB positive specimens and LJ culture detected 47.7%.Certain specimen types showed higher positivity rates of M.TB detection: synovial fluid (42.9%), pleural fluid (41.7%)by PCR analysis. ZN microscopy was associated with the low positivity rates for all specimens, the highest being 18.2% for sputum samples.

Conclusion: Tuberculosis PCR is a more rapid and reliable test in the diagnosis and management of tuberculosis. Both Pulmonary and extra-pulmonary specimens exhibit greater positivity rates by PCR analysis than by LJ culture and ZN smearing.

Keywords: Real-time, polymerase chain reaction, Mycobacterium, tuberculosis, pulmonary, extra-pulmonary, sensitivity, specificity.

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INTRODUCTION

Tuberculosis is an infectious disease and global burden with significant mortality and morbidity. TB is one of the top ten causes of death and millions of people suffer from TB each year. In 2017, an approximate 1.3 million died among HIV-negative population and an additional 300,000 deaths among HIV-positive individuals.[1] In 2015, over 10 million new cases of TB were reported globally. Among them 1.8 million resulted in deaths and over 95% were of poor countries.[1] In United States during the year 2018 the prevalence of pulmonary tuberculosis (PTB) was lowest ever according to an article published in the Centers for Disease Control and Prevention's (CDC) Morbidity and Mortality Weekly.[2] Pakistan ranks fifth globally among the 22 high-TB burden countries and contributes an estimated 63% of the disease towards the Eastern Mediterranean region. Annually around 430,000 people including 15,000 children contract tuberculosis in Pakistan and every year no less than 70,000 deaths can be attributed to the disease in the country. Pakistan is also estimated

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to have the fourth highest prevalence of multi-drug resistant tuberculosis (MDR-TB) globally.[3] Accurate and rapid diagnosis is critical to reducing such high infection and mortality rates. Diagnosis relies on a combined approach of clinical symptoms, chest X-ray, sputum smear microscopy, mycobacterial culture, and more recently, molecular methods.[4] Smear microscopy and culture are dependent on high numbers of mycobacteria for detection.[4] Smear microscopy is easy and simple test to perform; but it has low specification and sensitivity. [5,6] Mycobacteria culture usually takes 4-8 weeks, is prone to contamination and gives 20-30% of false negative results. [7,6,4] Nucleic acid amplification tests (NAATs) are increasingly considered as the standard diagnostic tool for TB and MDR-TB conferring improved sensitivity and rapid analysis.[6] Among NAATs real-time polymerase chain reaction give cost-effective results even in TB-negative patients.[8,9]

Extra-Pulmonary TB (EPTB) compromises of 15% to 20% of all cases of TB. Its global incidence has been increasing annually in the last decade with the rate of EPTB being >50% in HIV-coinfected patients. Because of the pauci-bacillary nature of EPTB it has always been difficult to demonstrate M. tuberculosis with routine tests. In 35% to 65% of the patients ZN staining or LJ culture are negative.[10] According to Amin et al. PCR could be a method of choice for identification of both pulmonary and extra-pulmonary tuberculosis.[11] PCR detects tuberculosis within few hours and it is very useful tool in diagnosing pauci-bacillary tuberculosis. Real time polymerase chain reactions are much better option than conventional PCR because of decreased risk of contamination and results become available in short time as compared to conventional PCR.[12] Early detection of Mycobacterium tuberculosis (MTB) DNA in clinical specimens consists of DNA extraction, amplification of target sequence. Different nucleic acid-based amplification techniques are used, commonly targeting the gene sequence of IS6110. IS6110, which is present as multiple copies in M.tuberculosis chromosome. The amplification of multiple copies gives more sensitive results as compared to amplification of single gene.[4] However, few studies from different geographical regions of the world have reported that some clinical isolates have either a single copy or no copy if IS6110 which leads to false negative results.[7] In this study, we evaluate the efficacy of ZN smear, LJ culture to real-time PCR of IS6110 sequence as gold standard in the detection of M. tuberculosis in respiratory and non-respiratory specimens.

MATERIAL AND METHODS

The study was conducted from January 2016 through December 2018 at Microbiology Department, of a tertiary care hospital of Peshawar, Pakistan. Ethical approval was sought from Research Ethics Review Committee. After a written informed consent, patients aged 2 to 85 years irrespective of gender with suspected TB based on history, clinical and radiological examination were registered for the study. Respiratory clinical specimens (including sputum, pleural fluid) non-respiratory samples (CSF, urine, synovial fluid, other fluids and tissue biopsies) were collected. Patients already diagnosed with pulmonary TB, repeat sample of the same patient, improperly collected samples and patients already on anti-TB treatment were excluded. All respiratory specimens were processed by the standard N-acetyl-L-cysteine and sodium hydroxide method with final concentration of NaOH as 2%. Tube containing digested and decontaminated specimens was centrifuged (3000 × g) for 15-20 minutes after which the supernatant was discarded and deposit was used for ZN staining, fluorescent staining and culture.ZN Staining: 2-3 drops from specimen were placed on a glass slide to prepare a smear before it was inoculated into MGIT 960 system. The smear was then placed into an oven at a temperature of 56ºC for about 5-6 minutes for drying, followed by the ZN staining. The number of AFB present was reported as; 1-9 bacilli/100 fields = 1+, 1-9 bacilli/10 fields = 2+, 1-9 bacilli/field = 3+ and more than 9 bacilli/field = 4+. Culture: All the specimens after being digested and decontaminated processed were inoculated into Lowenstein-Jenson Media and ZN staining was done for the confirmation of the presence or absence of AFB. LJ medium (HI media M168) was prepared as per manufacturer's instructions. M. tuberculosis H37Rv was inoculated on LJ as positive controls for M. tuberculosis. The media were incubated aerobically at 370 °C.

They were inspected daily for contamination for period of 10 days. After a week of incubation, the MB medium was tilted on alternate days for one week for first two weeks and thereafter once a week for inoculating the slant. Recovery of M. tuberculosis was the time of visible growth after inoculation. LJ medium showed the growth of typical buff colored, raised colonies of M. tuberculosis with rough surface PCR: Primers and probes were synthesized on the ABD394 DNA synthesizer (Applied Biosystems). Primers were derived from regions of the 16S rRNA gene that are conserved among mycobacterial species. The M. tuberculosis specific probe KY172-T3 (59-GGTGGAAAGCGCTTTAGCGGT-39) was chosen from a hypervariable region within the 16S rRNA gene. PCR amplifications were carried out in 100-ml reaction mixtures by adding 50 ml of template DNA or lysate to 50 ml of a premade amplification master mixture. Target DNAs were amplified in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer) as follows. A 2-min incubation at 50°C was followed by two cycles, each cycle consisting of 20 sec at 98°C, 20 sec at 62°C, and 45 sec at 72°C. This was followed by 35 cycles, each cycle consisting of 20 sec at 94°C, 20 sec at 62°C, and 45 sec at 72°C, for a total of 37 cycles. A final incubation at 72°C for ≥5 min was included to allow for completion of strand synthesis. Amplification products were detected by agarose gel electrophoresis

or hybridization to probe KY172-T3 in microwell plates. In the microwell plate assay, amplicons were denatured with 100 ml of denaturation solution. Denatured amplicons (25 ml) were added to wells of a microwell plate coated with probe KY172-T3. Hybridization was carried out at 37°C for 90 min in the presence of 100 ml of hybridization buffer. Detection of hybridized duplex was completed by using an avidin-horseradish peroxidase conjugate tetramethylbenzidine substrate system. Data were analyzed using computer statistical package of social sciences (SPSS) version 22.0. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each test was calculated. Fisher exact test was used to determine statistical significance of any association present between the variables.

RESULTS

Of the 293 samples, 165(56.3%) were from males and 128(43.7%) were of females. Mean (± SD) age was 44 (±20) ranging from 2 years to 85 years. Specimen types included: Cerebrospinal fluid, pleural fluid,sputum, urine, synovial fluid and other body fluids and biopsies (n=37). Frequency distribution is given in table 1. Only 3.1% (n=9) of specimens were ZN-smear positive for (MTB). LJ culture identified 7.2% (n=21) whereas PCR method detected (MTB) in 15% (n=44) of the total specimens. Smear microscopy correctly identified 20.5% of total (MTB) positive specimens whereas LJ culture detected 47.7%. Neither ZN microscopy nor LJ culture identified a truly negative patient as a positive patient therefore exhibit 100% specificity (see Table 2). Both tests did not give false positive results and thus exhibit 100% PPV.

However smear microscopy failed to detect 34 true positive patients and LJ culture also failed to detect (MTB) in 23 specimens thus both have low sensitivity and low NPV as opposed to PCR method.

Fisher exact test was performed to detect significance of association between specimen type, laboratory test and gender. LJ culture medium and ZN microscopy showed higher positivity rates for specimen types pleural fluid (41.7 %) and sputum (18.9%) respectivelyas opposed to other specimens (p-value =0.001). Positivity rates of specimen types showed significant association with PCRanalysis (p-value = 0.002).

Synovial fluid (42.9%), pleural fluid (41.7%) and urine (28.6%) samples exhibit greater positivity rates for (MTB) as opposed to CSF, other bodily fluids and biopsies. There is no significant difference between males and females in the detection of (MTB) by any test or specimen type (p-value >0.05).

Table 1: Frequency distribution of subjects and specimens.

Gender	Male	165(56.3%)
	Female	128(43.7%)
ZN smear	Positive	9(3.1%)
	Negative	284(96.9)
LJ culture	Positive	21(7.2%)
	Negative	23(92.8%)
PCR	Positive	44(15%)
	Negative	249(85%)
Specimen types	CSF	89(30.4%)
	Pleural fluid	12(4.1%)
	Sputum	44(15%)
	Urine	7(2.4%)
	Synovial fluid	7(2.4%)
	Other fluids	97(33.1%)
	Other biopsies	37(12.6%)

Table 2:Sensitivity, specificity and PPVs AND NPVs for each test:

	Sensitivity	**Specificity**	**Positive predictive value (PPV)**	**Negative predictive value (NPV)**
PCR	100%	100%	100%	100%
ZN staining	20%	100%	100%	88%
LJ culture	47%	100%	100%	91%

Table 3: Positivity rates of each specimen type:

		PCR Result		**LJ**		**ZN**	
		Positive	Negative	Positive	Negative	Positive	Negative
Specimen type	CSF	12(13.5%)	77(86.5%)	4(4.5%)	85 (95.5%)	0	100%
	Pleural fluid	5(41.7%)	7(58.3%)	5(41.7%)	7(58.3%)	0	100%
	Sputum	10(22.7%)	34(77.3%)	6(13.6%)	38(86.4%)	8(18.2%	36(81.8%)
	Urine	2(28.6%)	5(71.4%)	0	100%	0	100%
	Synovial fluid	3(42.9%)	4(57.1%)	2(28.6%)	5(71.4%)	0	100%
	Other fluids	11(11.3%)	86(88.7%)	4(4.1%)	93(95.9%)	1(1%)	96(99%)
	Other biopsies	1(2.7%)	36(97.3%)	0	100%	0	100%

DISCUSSION

In the local population where TB prevalence is high, our study has shown PCR performed better than the current routine diagnostic processes of ZN smear microscopy and LJ culture in detecting Mycobacteria tuberculosis in various specimen.

In this study, ZN showed least sensitivity (20%) of the 3 diagnostic methods concurrent with previous studies. Chakravorty et al found conventional smear method to have lower sensitivity (3.9%) which was increased to 21.1% by universal sample processing technique.[13]

Lydia et al reported ZN smearing to have higher sensitivity (50%) stating that ZN sensitivity being directly influenced by the HIV status of the patient on the type and quality of the specimen.[14] This is similar finding to our study as ZN mostly detected (MTB) in sputa as opposed to other specimens (p value <0.05). One of the reasons for low sensitivity is reported to be due to the fact that 104/ml is required for AFB to be seen using smear microscopy.[15,16]

In this research, LJ culture method demonstrated sensitivity of 47%. Chakravrty et al reported that conventional culture detected zero cases of MTB but universal sample processing culture method demonstrated 7.9% sensitivity.[14,13] In the past (MTB) culture as a gold standard with estimated sensitivity and specificity rates of 96% and 81%. However, a meta-analysis carried out in 2009, states (MTB) culture has limited value in clinical diagnosis as its sensitivity specificity rates have varied significantly from study to study.[11] A previous study in Pakistan reported a sensitivity rate of (MTB) culturing to be 15%-20% on over 50,000 specimens received from different geographical areas of the country.[17]

Our data revealed that PCR analysis showed 100% specificity and sensitivity. Bainomugisa et al showed PCR to have 100% sensitivity and 99% specificity.[18] A study conducted in Lusaka, using low-cost in-house one –tube nested PCR which showed 55% of sensitivity.[19] Cheng et al reported TB PCR to have overall sensitivity of 78.3% and a specificity of 100 %.[20]

In our study PCR positivity rates were higher in specimens such as synovial fluid and pleural fluid as opposed to other specimens. This is a statistically significant with a p-value of < 0.05. This may be because of larger volume of bodily fluid as opposed to that of sputa or other tissues specimens. This is similar finding to another study in Karachi where Amin et al reports PCR assay to demonstrate positivity rates of 70% in Bronchoalveolar lavage, Pleural fluid specimens.[11] This is concurrent with study by Chakravorty et al where PCR efficiencies were significantly high in samples of pleural fluid.[13]

CONCLUSION

Tests like ZN smear, culturing and PCR methods are used in diagnosis of TB. ZN staining is simple and fast test but has low sensitivity and specificity. Culturing tuberculosis has greater sensitivity but is time consuming, it takes many weeks to give results. PCR facilitates prompt detection of infectious agent in various specimen types, thus is appropriate for both pulmonary and extra pulmonary tuberculosis.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under

Tariq S: Conception, design of the work.

Khan M: Data acquisition, final approval

Tariq QUA: Data Collection.

Khan V: Analysis, interpretation of data.

Tariq N: Data analysis, typing.

Tariq H: Data analysis, editing.

Ahmad T: Bibliography

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.