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ROLE OF GEN-PROBE AMPLIFIED *MYCOBACTERIUM TUBERCULOSIS* DIRECT TEST (AMTD) IN THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS

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Abstract: A total of 717 specimens collected from nonrespiratory sites of patients suspected of extrapulmonary tuberculosis were tested for the presence of acid fast bacilli (AFB) by ZiehlNeelsen(ZN) staining, Conventional culture using Lowenstein Jensen's (LJ) medium and direct detection of *Mycobacterium tuberculosis* complex by the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (AMTD). The specimens included ascitic, pleural, pericardial, synovial fluids, abscess aspirates, lymph node aspirates, tissue biopsies, gastric aspirates, cerebrospinal fluid etc. All samples were processed by the *N*-acetyl-L-cysteine-sodium hydroxide decontamination procedure prior to testing. A total of 27 specimens were positive by any of the three methods; 5 samples showed acid-fast bacilli on ZN staining, and 27 samples were positive by AMTD, 12 of which were negative for *M. tuberculosis* complex by culture. After reviewing the patients' clinical charts to resolve discrepancies, the overall sensitivity, specificity, positive predictive value, and negative predictive value of AMTD was100, 100,100, and100% respectively. In conclusion, our results demonstrate that AMTD performs equally well with non respiratory specimens.

Keywords: AMTD, Extrapulmonary Tuberculosis, Culture, ZN stain

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INTRODUCTION

Tuberculosis continues to be a significant cause of morbidity and mortality worldwide, with 8 million new cases diagnosed anuually.[1] The sputum acid-fast staining and culture remain the gold standard for the diagnosis of active respiratory tuberculosis around the world; however,the former has been shown repeatedly to lack both sensitivity and, to some extent, specificity.[2] Since the mid 1990s a few commercially available molecular amplification assays have been introduced in an attempt to improve the accuracy and speed of detecting*M. tuberculosis* in clinical specimens.[3,4] The Amplified Mycobacterium Tuberculosis Direct (AMTD) test (Gen-Probe Inc., San Diego, Calif.) was initially approved by the Food and Drug Administration, Washington, D.C., in 1995. Subsequently a modification of AMTD was introduced in 1998, and it was approved by the Food and Drug Administration in September 1999.[5]The clinical diagnosis of extrapulmonary TB can be particularlychallenging, since samples such as pleural exudates and cerebrospinal fluids(CSFs) are known to contain only few mycobacteria, resultingin a low sensitivity of acid-fast staining techniques.Commercial nucleic acid amplification kits are licensed foruse with respiratory specimens only, and experience with specimensof nonrespiratory origin is still limited.The purpose of this study was to investigate whether theGen-Probe Amplified *Mycobacterium tuberculosis* Direct Test(AMTD) was suitable for a wide range of specimens other thanrespiratory secretions to establish early diagnosis of extrapulmonarytuberculosis.

MATERIALS AND METHODS

This prospective study was conducted in the Department of Microbiology, Dayanand Medical College and Hospital over a period of one year from Mar 2012 to Feb 2013.

Specimens: A total of 717non respiratory specimens(ascitic, pleural, pericardial and synovialfluids, abscess aspirates, and tissue and lymph node biopsy samples, as well asgastric aspirates and CSF samples) were obtained from patients visiting the OPD or admitted to various wards and ICUs of DMC & H, a referral hospital of North India. Of the 717 non respiratory specimens, majority of the sapmles obtained were CSF (51.2%) followed by GIT biopsies (12.7%), pleural fluid 99.9%), ascetic fluid (8.3%) and endometrial biopsies (7%) (Table- II). For most patients, there was a high suspicion of tuberculosis.

Concentration &Decontamination of Specimen: All theSpecimens including CSF and other body were concentrated &decontaminated by using the *N*-acetyl-L-cysteine and sodiumhydroxide (NALC-NaOH) protocol [6], keeping in view probable presence of pyogenic organisms. Briefly, an equal volume ofdigestant (3% NaOH, 1.45% sodium citrate, 0.5% NALC) was added to the specimen (up to 10 ml). After vortexing, the mixturewas shaken for 20 min, neutralized by adding sterile phosphate buffer saline, to a finalvolume of 50 ml, and centrifuged at 3,500*g* for

20 min. The supernatant wasdiscarded, and the sediment was resuspended in 2 ml of phosphate buffer saline. Half ofthe sediment was used to performAMTD and the otherhalf was inoculated onto culture media and used for acid-fast staining. Tissuebiopsy samples were homogenized beforeNALC-NaOH processing.The samples were stored at -20°C if there is any delay in processing.

Microscopy. Smears were stained with ZN stain.

Culture. Equal aliquots (approximately 300 µl) of the processed sedimentwere inoculated onto two solid slants, Lo¨wenstein-Jensen[7]anegg-based medium containing pyruvic acid and malachite green), and incubatedat 37°C for 8 weeks.All culture bottles were examined daily for the first 7days to detect rapid growers and to check the bacterial contamination. After that culture bottles were examined twice weekly till 8 weeks. Identification of mycobacteria was done on the basis of rate of growth, colony characteristics and ZN staining.

Amplified Mycobacterium Tuberculosis Direct Test (AMTD)

The Amplified Mycobacterium Tuberculosis Direct Test (AMTD)[8,9] detects specific *Mycobacterium tuberculosis* RNA by using an isothermal transcription-mediated amplification method. AMTD has been shown to be a sensitive, specific, and rapid method for detecting *M. tuberculosis* in clinical samples.The AMTD test was performed as per protocol given by suppliers of the kit. In brief, 50 µl of a decontaminated sample was added to 200 µl of specimen dilution buffer in a lysing tube, and the mixture was sonicated for 15 min in a water bath sonicator at room temperature to release the nucleic acids. For amplification, 25 µl of reconstituted amplification reagent was placed in a reaction tube and covered with 200 µl of mineral oil. Fifty microliters of lysate was transferred to the amplification tube, incubated at 95°C for 15 min, and cooled at 42°C for 5 min. An enzyme reagent mix (25 µl) was added, and the mixture was incubated at 42°C for 2 h. To terminate amplification, 20 µl of a termination reagent was added to each tube, and the mixtures were kept at 42°C for another 10 min. For detection, the reconstituted acridinium ester-labeled probe (100 µl) was added to the tubes, and they were incubated at 60°C for 15 min; then the selection reagent (300 µl) was added, and the mixtures were reincubated at 60°C for 10 min. All temperature-controlled incubation steps were carried out in heating blocks. Prior to being read in a luminometer (LEADER 50, Gen-Probe) the tubes were cooled at room temperature for 5 min. All runs included AMTD amplification positive and negative controls and hybridization positive and negative controls.

Interpretation:

≥500,000 RLU	Positive for *M. tuberculosis* complex rRNA
< 30,000 RLU	Negative for *M. tuberculosis* complex rRNA

30,000 to 499,999 RLU	Probable *M. tuberculosis* complex rRNA positive
	Repeat to verify results:
Repeat >30,000 RLU	Positive for *M. tuberculosis* complex rRNA
Repeat < 30,000 RLU	Negative for *M. tuberculosis* complex rRNA

Patients' clinical data. In case of any discordance between the test results for theAMTD and culture, the clinical charts of the patients wereevaluated. Clinical assessment included the patient's history, symptoms, chestX-ray, laboratory results, and follow-up observation as well as the results obtainedwith additional specimens from the patient that were sent to the mycobacteriologylaboratory.

RESULTS

A total of 717 clinical speceimen from extrapulmonary sites were included in the study. Only 5 samples were found to be positive for AFB by ZN staining, 15 were positive by conventional culture method (LJ slants) and 27 were positive for Mycobacterium tuberculosis complex by AMTD test. A total of 27 samples were positive for *Mycobacterium tuberculosis* complex by either of the methods. Percentage positivity of smear, culture and gene amplification was found to be 0.7, 2.1, 3.7 respectively (Table –I)

Out of 27AMTD positive samples 5 were smear positive and culture positive; 10 were smear negative and culture positive; 12 were both culture and smear negative. Out of the 690AMTD negative cases none were positive with smear or culture.

Table II depicts the sample wise distribution of all the 717 extrapulmonaryspeceimen tested. Maximum positive cases of extrapulmonary tuberculosis were of meningitis, as 14/27 mycobacteriom tuberculosis comlex positive speceimen were CSF, followed by ascitic fluid.

Conventional culture (LJ slants) detected 15 cases of tuberculosis, out of which 10 were missed on direct smear examination. Considering culture as gold standard, sensitivity of direct smear microscopy in extrapulmonaryspeceimen was only 33.33%, however specificity was 100%. Positive and negative predictive value of smear was 100 % and 98.61% respectively. However sensitivity of gene amplification was found to be 100% with a specificity of 97.9%. Positive and negative predictive value of gene amplification (AMTD) was evaluated as 55.56% and 100% respectively.

AMTD detected 27cases of extrapulmonary tuberculosis, out of which 12 cases were missed by conventional culture. After resolving the discrpient results on the basis of clinical history of the patients, all the 12 cases were confirmed cases of extra pulmonary tuberculosis. After

reviewing the patients' clinical charts to resolve discrepancies, the overall sensitivity, specificity, positive predictive value, and negative predictive value of AMTD was 100, 100, 100, and 100% respectively. Considering AMTD as gold standard for the diagnosis of extrapulmonary tuberculosis sensitivity specificity, positive predictive value and negative predictive value of culture was found to be 55.6%, 100%, 100% and 98.3% respectively.

TABLE I: POSITIVITY OF EXTRAPULMONARY SPECIMENS BY ZN SMEAR / CULTURE/ AMTD (N=717)

TECHNIQUE	Negative	Positive (%)
ZN smear	712(99.3)	5 (0.7)
LJ culture	702(97.9)	15 (2.1)
AMTD	690(96.3)	27(3.7)

TABLE II: Sample wise distribution of non respiratory specimen tested. (N=717)

Specimen	Number (%)	Smear Positive Number (%)	CulturePositive Number (%)	AMTDPositive Number (%)
CSF	371(51.7)	5 (1.3)	11(3.0)	14(3.8)
GIT Biopsy	92(12.8)	0	1(1.1)	3(3.3)
Pleural Fluid	72(1 0.0)	0	0	2(2.8)
Ascitic Fluid	60(8.4)	0	1(1.7)	4(6.7)
Endometrial Biopsy	51(7.1)	0	1(2.0)	2(3.9)
Tissue	25(3.5)	0	1(4.0)	1(4.0)
Pus	22(3.1)	0	0	1(4.6)
Urine	10(1.4)	0	0	0
Drain Fluid	5(0.7)	0	0	0
Pericardial Fluid	5(0.7)	0	0	0
Cystic Fluid	1(0.1)	0	0	0
Lymph Node Aspirate	1(0.1)	0	0	0
Synovial Fluid	1(0.1)	0	0	0
Total	**717**	**5(0.7)**	**15(2.1)**	**27(3.7)**

DISCUSSION

The diagnosis of mycobacterial infections remained practically unchanged for many decades and probably would have not progressed at all without the unexpected resurgence of TB which characterized the last twenty years of the 20th century.[10] Cultivation of *M. tuberculosis* from clinical samples is the gold standard for the diagnosis of active TB. It can detect 100 bacilli/ml of sputum in comparison with 5,000–10,000 bacilli/ml needed for microscopy.[11] However, further development is necessary for the more rapid, more reliable and less labour-intensive detection of mycobacteria, specifically in extrapulmonary specimens.

The Amplified Mycobacterium Tuberculosis DirectTest (MTD) detects *Mycobacterium tuberculosis* RNA by using an isothermal transcription-mediated amplification method with a sensitivity of96-100%, specificity 95-100% and is a rapid method (4-5Hrs) for detectionof *M. tuberculosis* in respiratory samples.[12-15]Since the introduction of AMTD forrapid detection of *M. tuberculosis* complex for respiratory samples, clinicians have repeatedly requested the testing of samplesother than respiratory secretions to accelerate the diagnosis ofextrapulmonary tuberculosis. As commercial kits are only licensedfor testing respiratory samples processed by NALCNaOHdecontamination, the present study was undertaken to investigate the suitabilityof the AMTD for specimens of nonrespiratory origin.

A total of 717 clinical speceimen from extrapulmonary sites were processed by AMTD and 27 were found to be positive (3.7%). Considering culture as gold standard, sensitivity of gene amplification was found to be 100% with a specificity of 97.9%. Positive and negative predictive value of gene amplification (AMTD) was evaluated as 55.56% and 100% respectively. In a similar study, the sensitivity and specificity of 84.6% and 98.7% respectively has been reported.[15]Further,Ayperan et al (2013) has reported a sensitivity, specificity, positive predictive value and negative predictiv value for AMTD Gen-Probe as 89%, 100%, 100% and 93%, respectively. [14]

In another study, a comparative analysis was made between three conventional tests (Ziehl–Neelsen staining, Löwenstein–Jensen culture, BACTEC mycobacteria growth indicator tubes) (MGIT) versus a DNA probe technique (AMTD), for diagnosis of tuberculosis (TB) , at a reference centre in Greece. Of a total of 136 TB patients diagnosed 133 of them (98%) were diagnosed by amplified MTD, 112 (82%) detected by the MGIT method, 102 (75%) by Löwenstein–Jensen culture and 75 (55%) by Ziehl–Neelsen staining. In addition using AMTD the results are ready within hours compared with days or weeks using conventional techniques.[9]In a series of 294 samples obtained from extrabronchial sites, the overall sensitivities and specificities of AMTD were shown to be as high as those reported by other investigators for

respiratory secretions.[8,16-19] Considering the various reports, the specificity of AMTD approached 100% [95,96] but its sensitivity ranged from 77.8%[20] to 92.8%.[21]

In the present study, AMTD detected 27 cases of extrapulmonary tuberculosis, out of which 12 cases were missed by conventional culture. After resolving the discrpient results on the basis of clinical history of the patients, all the 12 cases were confirmed cases of extra pulmonary tuberculosis. Therefore, culture cannot be considered the yardstick for the measurement of molecular biology test sensitivity and should be evaluated versus a "gold standard" built with clinical, radiological and biological findings.

Considering AMTD as gold standard for the diagnosis of extrapulmonary tuberculosis sensitivity specificity, positive predictive value and negative predictive value of culture was found to be only 55.6%, 100%, 100% and 98.3% respectively.

In conclusion, our study demonstrates the efficacy of AMTD in establishing the diagnosis of tuberculosis when samples other than respiratory secretions are assayed. The use of nucleic acid-based amplification techniques in the diagnosis of extrapulmonary tuberculosis may allow significantly earlier initiation of specific treatment.

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