Indian Journal of Microbiology Research 2020;7(3):273–280

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Indian Journal of Microbiology Research

Journal homepage: www.ipinnovative.com

Original Research Article

Detection of tuberculous meningitis by various microbiological modalities at a tertiary care hospital in north India

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ARTICLE INFO

Article history:
Received 01-09-2020
Accepted 02-09-2020
Available online 28-10-2020

Keywords:
intestinal TB
and CNS tuberculosis
that is
Tuberculoma without TBM.

ABSTRACT

Background & Objectives: Tuberculous meningitis is a common infection of the CNS, posing significant diagnostic and management challenges. Death in TBM patients is strongly associated with delay in diagnosis and treatment. Since, any single conventional or automated method for diagnosis of TBM has limited sensitivity; the main objectives of this study was – 1. Detect TBM by a combination of Direct Microscopy by ZN Staining, Culture by solid (LJ) and liquid media (BacT/Alert), besides GeneXpert (RT-PCR) in clinically suspected cases.; 2. Evaluate the role of GeneXpert for detection of Mycobacterium tuberculosis in CSF and demonstrate the sensitivity, specificity, PPV and NPV of GeneXpert in comparison with a set composite reference standard.

Materials and Methods: This was a prospective cross-sectional study carried out by the Department of Microbiology, GMC, Srinagar, from August '17 to September '19 CSF samples of 450 patients suspected of TBM were included in the study. CSF volume <0.1ml was excluded. A composite reference standard was devised which comprised of culture, ZN staining and empirical diagnosis. The samples were tested by ZN staining, solid culture, liquid culture and GeneXpert MTB/RIF using proper protocols. Patient details were obtained on a preformed proforma.

Results: Of the 450 suspected patients, 10% had Definitive TBM. The median age was 35 years with an almost equal male to female ratio. The sensitivities of ZN staining, Solid culture, liquid culture and Gene Xpert were 3%, 38%, 76% and 63% respectively. The specificity was 100% for all these tests, 95% CI being, 99-100%. The NPV was 84.02%, 100%, 91% and 93.6% whereas, the PPV was 84.89%, 100%, 100% and 99.1% respectively. Rifampicin resistance was positive in 2%. 95% cases had a very low bacillary load and 2.2% patients showed low and medium bacillary load. 12% patients had concomitant pulmonary TB and 10% with other forms of extrapulmonary TB.

Conclusion: Gene/Xpert as a frontline diagnostic tool, is a game changer. It abridges presumptive prescribing of tuberculosis treatment. Speedy detection of TBM especially in smear negative cases and detection of drug resistance in the paucibacillary CSF improves outcome for patients with TBM /MDR TBM. However, additional tests, with better sensitivity are required and we must make the best use of the tests we have by testing adequate volumes (5 -10ml) of CSF.

1. Introduction

According to the Global Tuberculosis report 2014 of World Health Organization (WHO), Tuberculosis (TB) is one of the most common communicable and deadliest disease caused by the Mycobacterium tuberculosis (MTB).[1] The disease mainly involves the lung tissue (pulmonary TB) and is an airborne disease.[2] In 2013, out of the estimated global annual incidence of 9 million TB cases, the incidence of TB in India was 2.1 million (24%) cases/year (one fourth of global incidence).[3]

False-negative results and misdiagnosis of TB suspects are common and is attributed to poor sensitivity of Ziehl-Neelsen (ZN) smear microscopy which is used for its initial diagnosis.[4,5] The gold standard for diagnosis of

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https://doi.org/10.18231/j.ijmr.2020.049

Mycobacterial TB is culture, but it is slow and takes a lot of time (around 2-6 weeks-time) to yield a final result.[1,4,5] In the recent times, a number of Nucleic Acid Amplification (NAA) methods have been introduced for rapid detection and identification of MTB in clinical specimens of pulmonary and EP-TB cases. The major advantages of these techniques are the rapidity of diagnosis and detection of small quantities of MTB genomic copies in a sample.[6]

The GeneXpert is a DNA-PCR technique which simultaneously detects Mycobacterium Tuberculosis and resistance to rifampicin medication.[7–9] It is the first fully automatic cartridge based nucleic acid amplification (CB-NAAT) assay for tuberculosis and it gives results within 2 hours.[9,10] The diagnostic sensitivity and specificity of CB-NAAT for pulmonary tuberculosis is high.[9,10] Patients with high risk of tuberculosis like presumptive HIV-associated TB patients and pediatric presumptive including extra pulmonary cases in whom AFB smear examination is usually negative, are the most likely to be benefited from GeneXpert.[3,10]

We sought to evaluate the various methods of detection of Tuberculous meningitis, and the diagnostic accuracy of Gene XPERT MTB/RIF for detection of tuberculous meningitis in our set up.

2. Aims and Objectives

1. To diagnose Tuberculous Meningitis by combination of Direct Microscopy by ZN Staining, culture by conventional solid (LJ Medium) and liquid (BacTAlert) and GeneXpert (RTPCR) in clinically suspected cases.
2. To evaluate the role of GeneXpert for detection of Mycobacterium tuberculosis in CSF and demonstrate the sensitivity, specificity, positive predictive value and negative predictive value of GeneXpert in comparison with a set composite reference standard.

3. Materials and Methods

A total of 450 CSF samples, from August 2017 to September 2019, were analysed in this cross-sectional, prospective study carried out in the Mycobacteriology laboratory of Postgraduate Department of Microbiology, Government Medical College, Srinagar.

The ethical clearance was obtained from the Ethical Clearance Committee of Government Medical College, Srinagar.

3.1. The specimen

CSF.

3.2. Inclusion criteria

CSF of patients suspected of having Tuberculous meningitis on clinical evaluation.

Samples from patients falling under the inclusion criteria of the study and manifesting any symptoms and signs of Tuberculous meningitis were included in the study.

Volume of CSF more than 0.1 ml.

3.3. Exclusion criteria

Volume of CSF less than 0.1 ml.

3.4. Sample collection and transport

Aseptically collected CSF sample from cases suspected of having tuberculous meningitis by lumbar puncture were transferred into 4 sterile collection tubes without any additives. The sample was delivered immediately to the laboratory and stored at room temperature till further processing. Tube 1 was used for chemistry studies, glucose and protein count. Tube 2 was used for Microbiological studies whereas Tubes 3 and 4 were used for cell counts and differential.

A minimum of 5 to 10 ml of CSF is ideal to be sent for detecting mycobacteria.

3.5. Sample processing

The Preferred processing method for CSF depended on the volume of sample available for testing.

For more Than 1ml of CSF:

1. Upon receipt in the TB laboratory, The CSF sample was centrifuged.
2. CSF was transferred in a conical centrifuge tube/falcon tube and concentrated at 3000g for 15 minutes.
3. The supernatant was carefully poured off through a funnel into a discard can containing 5% phenol.
4. Decanting of concentrated CSF was performed within a BSC to leave a 0.5-ml sediment which was then used for making smears for Ziehl Neelsen staining(0.1ml), inoculation on to the LJ medium (0.1ml), Bactalert bottle (0.1ml) and GeneXpert MTB/RIF test (0.2ml).

For less Than 1ml of CSF

The CSF sample was used directly was used for making smears by concentration method for Ziehl Neelsen staining, inoculation on to the LJ medium and GeneXpert MTB/RIF test.

For less Than 0.1ml of CSF

The sample was rejected as it is insufficient for testing.

The following precautions were taken:

1. Apron, gloves and facemasks were worn while dealing with mycobacteria.

2. Aerosol production by doing vortex and mixing in tightly capped containers was avoided.
3. Aerosol generation by spluttering or vibration of the loop was avoided.
4. The charged loop was dipped in a container having alcohol and sand mixture before heat sterilization when flame was used, so as to prevent the formation of aerosols.
5. The air and exhaust of the safety cabinet was run for an additional time (at least 5 min) after completion of A.F.B. work.

3.6. Devising up the reference standard

For evaluation of a diagnostic test culture alone is not an optimal reference standard for TBM, owing to the paucibacillary nature of the disease. And therefore, a composite reference standard was set for comparison of GeneXpert MTB/RIF which comprised of the following investigations:

1. Culture (LJ
2. Microscopy(ZN
3. Empirical Diagnosis based on:
   a. Clinical features
   b. Biochemical laboratory parameters for aiding in the diagnosis of TBM included
   i. CSF analysis
   ii. Adenosine deaminase levels:
   c. Radiological findings
   i. Chest X-ray
   ii. Neuroimaging:

The patients presumed to have Tuberculous meningitis were assessed in the IPD and detailed history including age, sex, socio-demographic profile, clinical history emphasising on presence of fever, headache, vomiting, altered sensorium was taken. They were clinically evaluated for signs of meningeal irritation, raised intracranial pressure, cranial nerve deficits, focal neurological deficits, any co morbid illness, evidence of TB elsewhere in the body and past history of TB. All these were noted in the prescribed proforma. Uniform case definition was applied to all the suspected cases. The diagnosis of TBM was confirmed if AFB were seen or cultured from the CSF or positive on geneXpert, was probable if AFB were found from another site or there was evidence of active extraneural tuberculosis, and was possible if the history was longer than 5 days and the CSF abnormalities included a raised white cell count, predominantly lymphocytes, and low CSF/blood glucose ratio. The diagnosis of TBM was excluded if another pathogen was seen or cultured from the CSF or if the patient recovered without antituberculosis chemotherapy.

Socio-demographic, clinical information and laboratory results of participants were crosschecked before being entered into computer software. Categorical variables were summarized as frequency and percentage. Continuous variables were summarized as mean and standard deviation. Data analysis was done by using EpiInfo 7.0. The data was entered in MS Excel spreadsheet and the categorical variables were summarized as frequencies and percentages. EpiInfo 7.2 was used to evaluate the validity parameters. Sensitivity, specificity, PPV, NPV and diagnostic accuracy was reported as percentages along with their 95% confidence interval. Two sided p-values were reported and a $p<0.05$ was considered statistically significant.

4. Observations and Results

During the study period, 450 eligible patients were included in the analysis. Uniform case definition was applied, and 45 were finally classified as “definite TBM”, 24“probable TBM”, 2 “possible TBM” and 379 “not TBM” (Chart 1).

26 Cases were diagnosed clinically and treated for TBM by the clinician, these were the cases that were missed by microbiological diagnosis (false negatives). They were labelled as clinically diagnosed TBM (Probable TBM/Possible TBM) cases and were put on anti-tubercular chemotherapy.

Table 1: Agedistribution of TBM

Age	No. of Patients	Percent
<=20	3	4%
21-40	29	41%
41-60	24	34%
61-70	7	10%
>70	8	11%

Most common age group in our study was between 21 to 40 years, and those aged >70 years comprised only 5.7%. 16% of patients were in age group of 31-40.

Slightly More than half (51%) patients were males while as female comprised of 49%. Male to female ratio being 1.2:1.

Majority of the patients, presented with fever, headache, vomiting and altered sensorium (32%).

30% patients had fever, headache and vomiting. 9% of patients showed focal neurological signs such as weak limb. Diplopia/Cranial nerve paresis was present in 5% of our TBM patients. 2% patients presented with slurred speech.

Table 2: The Diagnostic evaluation of Microscopy(ZN) for TBM

Parameter	Estimate	95% confidence interval
Sensitivity	2.86	0.79-9.83
Specificity	100	99-100
Positive Predictive value	84.82	81.2-87.85
Negative predictive value	84.89	81.29-87.9

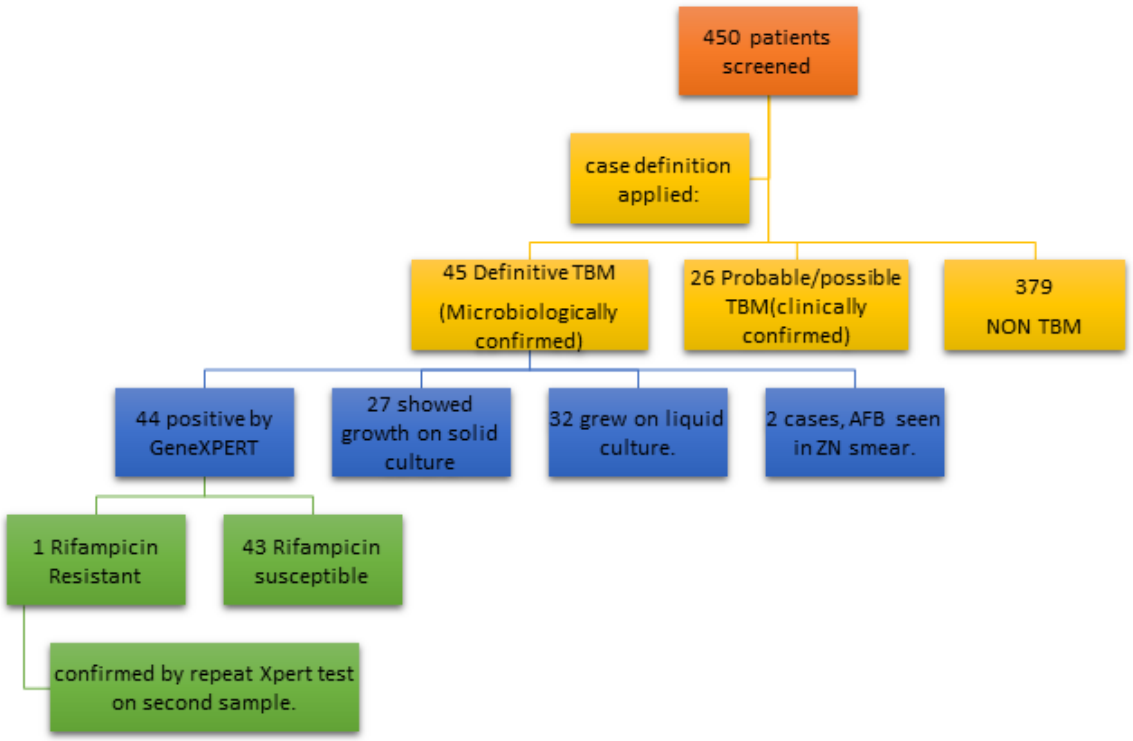

Chart 1:

After All the 450 samples were subject to ZN staining, only in two CSF samples, AFB were seen.

The sensitivity of ZN staining for detection of MTB in CSF is very less, 2.86%. In contrast, the specificity is 100%. The positive predictive value and negative predictive values are 100% and 84.89% respectively.

Table 3: The Diagnostic evaluation of Solid Culture for TBM

Parameter	Estimate	95% confidence interval
Sensitivity	38%	27 to 49
Specificity	100%	99 to100
Positive Predictive value	100%	99 to 100
Negative predictive value	89.93%	78 to 100

All the 450 Patients were subject to culture on LJ media, out of which 27 were positive Most of them were positive after the 4^{th} week and two by the 6^{th} week.

The sensitivity of Solid culture LJ media for detection of MTB in CSF is around 27 to 49% whereas the specificity is 100%. The positive predictive value and negative predictive values are 100% and 89.9% respectively.

The limitation of our study was that liquid culture was done only for 30% of the patients.

Table 4: Solid V/s liquid culture for MTB in CSF

Media	Growth	Total Samples	Percentage
Solid culture	27	450	6%
Liquid culture	32	135	24%

Liquid culture detected around 32 cases of TBM out of the 135 suspected cases that it was put for, which makes around; 24%. The detection rate of MTB from CSF by liquid culture is higher than that of solid culture. It detected MTB in 5 more TBM cases that solid culture missed.

Table 5: The diagnostic evaluation of liquid culture for TBM

Parameter	Estimate	95% confidence interval
Sensitivity	76.2%	60.5 to 87.9
Specificity	100%	96 to 100
Positive Predictive value	100%	89 to 100
Negative predictive value	91.2	84 to 95

Liquid Culture was evaluated with respect to GeneXpert that resulted in a sensitivity of 76% and specificity 100%. The negative predictive value and positive predictive value

was 91.2% and 100% respectively.

Table 6: Results from gene expert

MTB detection	No. of Samples	Percentage
Negative	406	90.22
Positive	44	9.78
Grand Total	450	100

9.78% of the clinically presumed TBM cases were positive for MTB by geneXpert MTB/RIF assay 2% patients positive for MTB by GeneXpert MTB/RIF assay were resistant to Rifampicin.

The bacillary load of 44 positive patients by GeneXpert MTB/RIF was documented. 95% of the cases had very low bacillary load owing to the paucibacillary nature of the disease and 2.2% patients showed low and medium bacillary load. Xpert did not report a high bacterial load for any CSF sample.

Table 7: The diagnostic evaluation of GeneXpert MTB/RIF for TBM

Parameter	Estimate	95% confidence interval
Sensitivity	63%	52 -74
Specificity	100%	99, 100[1]
Positive Predictive value	100%	99, 100[1]
Negative predictive value	93.6%	90.78, 95.59[1]

All the 450 suspected TBM CSF samples were subjected to GeneXpert Testing, out of which 44 samples were positive for MTB.

The sensitivity of GeneXpert against the composite reference standard is 63% whereas the specificity is 100%.

The positive predictive value and the negative predictive value are 100% and 93.6% respectively.

GeneXpert detected 63% of the patients of definitive TBM in our study.

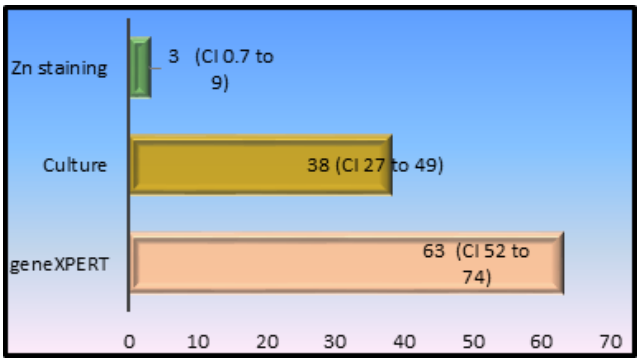

Fig. 1: Sensitivities (with confidence intervals) of ZN smear, solid culture and Xpert MTB/RIF against composite reference standard for the diagnosis of TB meningitis in all patients

4.1. Venn diagram

The number of patients with a clinical diagnosis of TBM with a positive test result for each of the modalities used, ZN smear, culture and Xpert MTB/RIF respectively.

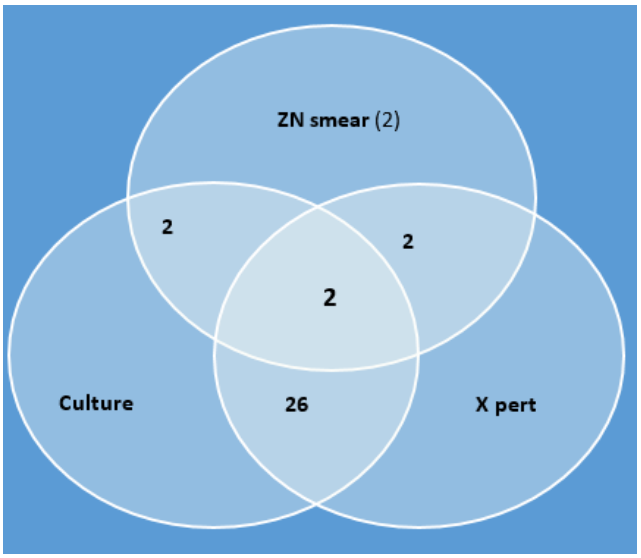

Fig. 2: Venn diagram of positive test result by diagnostic technique Number (N) of total positive test results are noted in brackets. Number of shared positive results are shown in bold type. ZN=Ziehl-Neelsen

In TBM group, mean CSF cell count was 300 cells/mm^3, with lymphocytic pleocytosis**,** mean CSF protein and glucose were 180 mg/dl and 35 mg/dl respectively. Statistically significant results were obtained between TBM and Non-TBM when CSF features of protein >100mg%, cells>20/mm3 & CSF sugar <0.5 of corresponding blood sugar were compared.

In our study, not all patients underwent ADA level testing Out of our 71 TBM patients, 20 had undergone testing for CSF ADA. Increase in ADA levels helped to add to the diagnostic algorithm in two patients, who were clinically diagnosed with levels more than 20 IU.

Bilateral infiltrates and hilar adenopathy (suggestive of active tuberculosis) and miliary mottling seen in 9 cases of our TBM patients.

Out of our 71 TBM cases, (microbiologically confirmed as well as clinically diagnosed), 9% of patients had concomitant pulmonary TB (including pulmonary infiltrates as well as miliary TB) and 7% had Extrapulmonary TB.

The extrapulmonary TB included 2 cases of Spinal TB (Potts disease), 1 case each of lymphadenitis, lupus vulgaris, renal TB, intestinal TB, and CNS tuberculosis, that is, Tuberculoma without TBM.

2 Patients out of 71 TBM patients passed away.

5. Discussion

Most common age group in our study was between 21 to 40 years, with a median age of 35 years and an almost equal male to female ratio. In a report by Roca et al, Spain the median of age of their TBM patients was 34 years, and 59% were males.[11] Saleem et al, Kashmir in their report on TBM, in 2004, mentioned a female predominance of 59% and the most common age group was 29 to 30 years.[12]

Around 5000 to 10,000 organisms per mL must be present in the specimen for TB bacteria to be visible by microscopy.[13] Kent et al[14] and Marais et al,[15] stated that, the sensitivity of smear microscopy is generally low; 2% to 30% in CSF.[14,15] In contrast, the specificity of smear microscopy tends to be quite high, $\geq$ 90%.[14,15]

The low sensitivity of CSF smear examination was not surprising for our report, the sensitivity of ZN staining for detection of MTB in CSF was 2.86% and the specificity was 100%. This is because the number of bacilli present in the CSF is never large, the volume of CSF obtained at tap for examination was small most of the times and the fraction viewed through an oil immersion lens is miniscule.

Thwaites et al,[16] in their study, established that both CSF volume and duration of the microscopic evaluation are independently associated with bacteriological confirmation of CNS tuberculosis, suggesting that a minimum of 6 ml of CSF fluid should be examined microscopically for a period of 30 min and can improve the yield to more than 60% of clinically diagnosed cases.[16]

Early studies by Kennedy et al[17] demonstrated that acid-fast stains can detect up to higher number of cases, although results are highly dependent on CSF volume, timeliness of sample delivery to the lab and analysis, and the technical expertise of lab personnel.[17]

Mycobacterial culture is a method used to grow bacteria on nutrient-rich media. In comparison with microscopy, a positive culture requires only around 100 organisms per mL and therefore can detect lower numbers of TB bacteria.[13] Additionally, culture is essential for species identification and DST.[18] The sensitivity of Solid culture LJ media for detection of MTB in CSF in our study was around 27 to 49% whereas the specificity was 100%. The positive predictive value and negative predictive values were 100% and 89.9% respectively. Van well[19] mentioned in their work, that the culture has sensitivity, 18%-83% but is highly specific (100%).[19] Several case series also established CSF culture sensitivities of 25 to 70%.[20]

Liquid culture of M. tuberculosis is considered the gold standard for diagnosis. The limitation of our study was that liquid culture was done only for 30% of the suspected TBM patients, due to the paucity in volume of CSF sent to the department of microbiology as well as cost constraints. Liquid culture detected around 32 cases of TBM out of the 135 suspected cases that it was put for, which makes around; 23%. The sensitivity and specificity of liquid culture was evaluated keeping GeneXpert as the reference standard. Liquid culture showed a sensitivity of 76% and specificity of 100%.

Liquid culture provides results twice as faster than the solid culture.[21] Therefore, liquid culture provides a tremendous advantage over LJ medium in terms of its rapidity in growth detection, increased sensitivity and a lower rate of specimens lost due to contamination.

Nevertheless, due to the slow growing nature of mycobacteria, the time to a positive result may range from 2-8 weeks. This renders the test inefficient for clinical decision-making regarding treatment initiation, although a positive result can confirm the decision to continue therapy and provides an isolate for drug susceptibility evaluations. Yet again, increase in CSF volume is required to improve the sensitivity.[22]

The overall sensitivity of GeneXpert in our report was 63% (52 to 74%); compared to the composite reference standard.[22] A bacteriological standard is too insensitive to be used alone for evaluation of new diagnostic tests, which may be more sensitive than culture and Ziehl-Neelsen.[22] Therefore, like other investigators we used composite reference standards for diagnosis that use culture, Ziehl-Neelsen, clinical, biochemical and radiological findings.[15] Use of such reference standards may better reflect the clinically relevant population, rather than a selected subpopulation with high bacterial burden.[15,22] The specificity of Xpert was 100%. The positive predictive value and the negative predictive value were 100% and 93.6%, respectively.

Our True-positive patients benefited from rapid diagnosis and appropriate treatment. True-negative patients were excluded and spared from unnecessary treatment. We had no False-positive patients by Xpert, who would otherwise experience anxiety and morbidity caused by additional testing, unnecessary treatment, and possible adverse effects; possible stigma associated with a TB diagnosis; and the possibility that a false positive may have deterred the physician from further diagnostic evaluation. The Negative predictive value of 89% implied that Xpert MTB/RIF test was useful in determining that a patient has TB meningitis but was not useful in determining that a patient does not have TB meningitis. A positive Xpert MTB/RIF assay result does not imply viability of the organism and, thus cannot be used to monitor response to treatment, treatment success, treatment failure, or relapse.[15,22]

The diagnostic evaluation of Xpert reported in our study is almost corresponding with the report by Nguyen et al. (2014) with 59% sensitivity and 99% specificity in their study.[23] Wang (2016) China, in their study showed sensitivity analogous to our report that is, 62% and a specificity of 99%.[24] Bahr et al (2015), had a 60% sensitivity and 97% specificity in their study.[25] The sensitivity and Negative predictive value remain inadequate

for use of Xpert as a rule out test. The sensitivity is low as the pathogen load is below the detection limit of the assay. At least 131 bacilli should be present of the assay to be positive. The Xpert MTB/RIF test system depends upon capture and lysis of whole bacilli[26] and therefore, as for other microbiological tests for TBM, high volumes (>7mls) of CSF are thought to be crucial to obtaining high sensitivity.[22] Xpert Assay sample reagent has better homogenisation and liquefaction efficiency and hence more sensitivity than solid culture. Furthermore, GeneXpert can amplify DNA from dead bacilli, which could be another reason of culture being negative in some of the MTB positive samples by Xpert.[22,26]

The reason for false-negative GeneXpert result may be due to a very low bacillary count in the CSF sample. Another explanation might be that the GeneXpert has a low NPV. As recommended by the WHO, patients suspected of having TBM who receive a negative GeneXpert result should undergo further diagnostic studies.[15]

The GeneXpert provides a semi-quantitative estimate of the concentration of bacilli present in a clinical sample. The qualitative estimation of bacterial load by GeneXpert in our study showed that 95% of the cases had a very low bacillary load and 2.2% patients showed low and medium bacillary load. Xpert did not report a high bacterial load for any CSF sample. In the study carried out by Nhu et al,[27] the majority results were categorized by Xpert as very low (54/109; 49.5%) or low (46/109; 42.2%), with 9 medium results (8.3%) with none being high. The very low bacillary load for all most all patients in our study owes to the paucibacillary nature of the disease[27]. The CSF with medium bacillary load was also positive on microscopy.

Rare false-positive results for rifampicin resistance have been reported with Xpert,[28] and the consequences of mistakenly treating a patient with rifampicin-susceptible TBM with weak second-line regimens would be grievous.

2% of our (44) definitive TBM patients tested by GeneXpert MTB/RIF assay were resistant to Rifampicin. The resistance was further confirmed by a repeat CBNAAT test on a second specimen from the same patient. In the report by Nhu et al,[27] Rifampicin resistance was detected in four cases during their study by Xpert MTB/RIF.

Xpert detects rpoB mutations, which are present in only 95% of phenotypically rifampin-resistant M. tuberculosis isolates.[29] The mutations in isoniazid occur at rate of 1 in $10^6$and for Rifampicin the mutations occur at the rate of 1 in 10^8. Implying that, there will be one Isoniazid resistant isolate in 10^6bacilli and one Rifampicin resistant isolate in 10^8bacilli. So by the time mutations occur in Rifampicin, mutations will have already taken place in Isoniazid. Hence, RIFAMPICIN is considered the surrogate marker for detection of MDR in MTB.[30] For patients with rifampicin resistance detected by Xpert MTB/RIF and a clinical suspicion of MDR TBM, second-line drugs with appropriate CSF penetration should not be withheld until the results from conventional DST become available.[29,30]

The CSF biochemical picture was consistent with the usual observation of CSF findings suggestive of TBM seen in various studies. In our TBM group, mean CSF cell count was 300 cells /mm^3, with Lymphocytic Pleocytosis in 80% of cases, mean CSF protein and glucose were 180 mg/dl and 35 mg/dl respectively. Statistically significant results were obtained between TBM and Non TBM when CSF features of protein >100mg%, cells >20/mm3 & CSF sugar <0.5 of corresponding blood sugar were compared. In the study, carried out by Christensen et al., 86% had elevated protein values, 90% had elevated WBC count and 50% patients had CSF: blood glucose ratio of 100mg/dl and CSF sugar less than 60% of corresponding blood sugar.[31]

The low mortality in our TBM cases is perhaps due to the rapid detection by GeneXpert, timely Diagnosis and treatment. Including Genexpert in the diagnostic algorithm has definintively improved outcome of TB patients during these years by its speedy detection. Among the patients that missed out on GeneXpert, alternate diagnostic tests, especially MRI has helped.

6. Conclusion

1. Early diagnosis and treatment with effective anti-tuberculosis drugs remains the most crucial aspect of management of this devastating condition. Thus, we conclude by our study that:-
2. GeneXpert MTB/RIF was the best diagnostic modality for detecting TBM amid solid culture, ZN staining and clinical diagnosis of smear-negative TB.

7. Source of Funding

None.

8. Conflict of Interest

None.

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Cite this article: Iqbal I, Farhana A, Zahoor D, Bashir H. Detection of tuberculous meningitis by various microbiological modalities at a tertiary care hospital in north India. *Indian J Microbiol Res* 2020;7(3):273-280.