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Study of Usefulness of CBNAAT in Clinically Suspected Tuberculosis Patients of Age 6 Months To 12 Years at A Tertiary Care Hospital - A Hospital Based Prospective Study

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Abstract

Background: Globally, tuberculosis is one of the top ten causes of death among paediatric age group. As tuberculosis is a global problem, for eradication of the disease, early diagnosis, timely identification and improved detection is essential. However, the diagnosis of tuberculosis is challenging in cases of insufficient sputum and paucity of bacilli. With this background, present study was planned to compare the effectiveness of cartridge-based PCR method (CBNAAT) with the conventional methods (acid-fast staining and Auramine Phenol Fluorescent staining) in the diagnosis of paediatric tuberculosis.

Method: A total 114 children of age 6 months to <12 years presented with symptoms suggestive of tuberculosis were studied during a period from 1 January 2020 to 31 DEC 2020.

Results: The overall detection rate of tuberculosis was 5.3%, which was higher in male children <5 years of age. The detection rates using acid-fast staining and fluorescent staining were 1.05% and 3.15% respectively while the detection rate using CBNAAT was increased to 5.3%. Acid-fast staining had sensitivity of 16.6% and specificity of 100%. Positive and negative predictive value was 100% and 95.6% respectively. Fluorescent staining had sensitivity of 50% and specificity of 100%. Positive and negative predictive value was 100% and 97.3% respectively. CBNAAT had more sensitivity and specificity when compared with other methods.

Conclusion: Thus, CBNAAT was advantageous as it could detect more cases which are missed by other conventional methods. It gives rapid results in two hours and also determines resistance to rifampicin simultaneously. Hence, we recommend the use of CBNAAT in the routine diagnosis of paediatric tuberculosis.

Keywords: Paediatric Tuberculosis; Cartridge-based PCR; Acid-fast staining; Auramine Phenol Fluorescent staining; Sensitivity; Specificity

Introduction

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis. In 2019, approximately 10 million TB cases detected globally, among them 20% of individuals were from India [1, 2]. According to WHO estimates, tuberculosis affects around 5,30,000 children and about 74,000 deaths were reported among them [3]. However, a systematic review of 97 studies conducted all over the world estimated that the actual burden of paediatric tuberculosis was around one million [4]. Such an enormous difference is due to under reporting of cases particularly among developing countries. The increased death rate of this curable disease is due to lack of ideal diagnostic methods and clinical criteria.

WHO has advocated the use of newer molecular techniques like CBNAAT and Line probe assay for the diagnosis of tuberculosis in adults. However, these methods have not been validated in children. Microscopy is still used as the initial method for

diagnosing tuberculosis in developing countries. Paediatric tuberculosis, owing to its paucibacillary nature and difficulty in collection of samples makes the diagnosis difficult by these conventional methods. Culture, although being gold standard is not used routinely due to delay in results and availability of investigation [5].

Despite the discovery of many newer methods in the diagnosis of tuberculosis, a robust point of care test is required for effective management of Paediatric tuberculosis. CBNAAT has now become a boon in the early and prompt diagnosis of tuberculosis especially in paediatric TB cases and extrapulmonary TB cases. The CBNAAT besides providing faster results also detects resistance to rifampicin simultaneously. It is now being used as a first line of investigation in HIV–TB coinfection and in extrapulmonary tuberculosis. Newer generations of CBNAAT are in the pipeline to create a low cost near patient technology for the diagnosis of tuberculosis [6]. Hence the aim of present study was to compare the CBNAAT with the conventional methods for a better and earlier diagnosis of paediatric tuberculosis. The purpose is to fill the lacunae and resolve the difficulties in the diagnosis of paediatric tuberculosis.

Materials And Methods

After obtaining Institutional Ethical Committee approval and written informed consent from the parents/guardians, this prospective observational study was conducted in the Department of Paediatrics at rural tertiary care hospital in central India during a period of 12 months from 1 January 2020 to 31 DEC 2020. A total 114 children of age 6 months to less than 12 years with symptoms of persistent fever and/or cough for more than two weeks, loss of appetite or unexplained weight loss/ no weight gain in the past three months, who has history of contact with TB case, children with significant superficial lymphadenopathy, severe respiratory distress and chest X-Ray suggestive of tuberculosis and children with failure to thrive symptoms were included in the study. Exclusion criteria were patients of age less than 6 months or more than 12 years with symptoms of tuberculosis, who was a known case of tuberculosis and on anti-tubercular treatment and parents/ guardians not giving consent to participate in the study.

Samples were collected from children based on their complaints after obtaining their parent's or guardian's consent for the same. Specimens included gastric aspirate, sputum, induced sputum, tracheal aspirate, ascitic fluid, pleural fluid, lymph node aspirate and CSF. All the specimens were processed- The preparation of NALC-NAOH solution [7], preparation of phosphate buffer [7], procedure for digestion and decontamination of samples [7], processing of sterile samples [8] and processing of tissue samples [8] were done.

The following procedure was followed for preparing smears of sterile samples – pleural fluid, ascitic fluid and lymph node aspirate.

1. One loopful of the sample was kept over the slide and allowed to air dry.
2. One more drop was placed over the same spot and allowed to dry.
3. Another drop of the centrifuged sample was placed on the same spot, air dried and fixed.
4. For other specimens, preparation of smear was done in the following manner: -
5. The purulent part of the specimen was taken and spread over the slide for 3×2 cm evenly.
6. The smear was allowed to air dry at room temperature and then fixed by passing through the flame.

Acid Fast Staining:

Preparation of reagents:

Acid fast carbol fuchsin [7]: Basic fuchsin – 4 grams; Carbolic acid (phenol) – 8 grams; 95% alcohol – 20 ml and Distilled water – 100 ml

Acid/ Alcohol (Decolouriser): 3% Hydrochloric acid– 3 ml and 95% Ethanol – 97 ml

Methylene Blue (Counterstain): Methylene blue– 0.3 grams and Distilled water – 100

Procedure

The prepared slides were kept over the staining rack. Slides were flooded with Acid fast's carbol fuchsin and left for 8 minutes. Then slides were rinsed with water until the stain gets washed away. Slides were decolourised with acid/alcohol for 2 minutes and then washed thoroughly. Methylene blue solution was added and left for 3 minutes. The slides were rinsed with water and kept for air dry. The slides were examined under 100X oil immersion objective lens.

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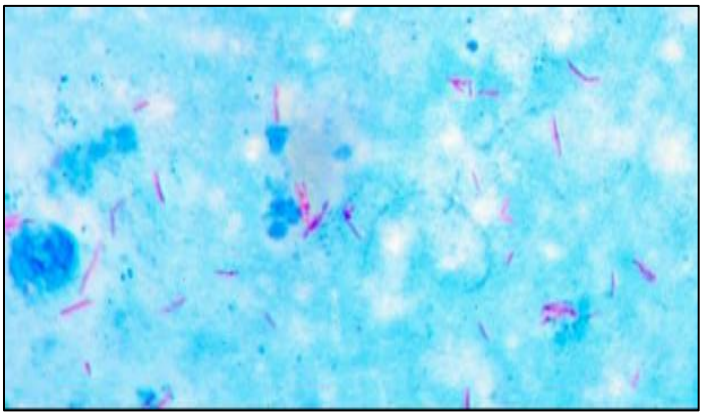
The slides were systematically scanned in a zig-zag manner covering the whole smear from left to right. The smear was graded according to the NTEP guidelines, (Table 1).

Table 1: Grading of Smears [9]

	No. of fields to be examined	Grading	Results
No AFB in 100 fields	100	0	Negative
1-9 AFB/ 100 fields	100	Scanty	Positive
10-99 AFB/ 100 fields	100	1+	Positive
1-10 AFB/ field	50	2+	Positive
>10 AFB/ field	20	3+	Positive

Figure 1: Microscopic view showing rod shaped M TB bacilli after ZN stain



Auramine Phenol O Fluorescent Staining

1) Preparation of reagents [10]:

Auramine Phenol O: 40 ml of phenol and 60 ml of glycerine were mixed in 1000 ml flask; 3 grams of auramine was added to the solution; 900 ml of distilled water was added to the mixture with constant stirring; The solution was kept in dark undisturbed for 4 days; The solution was filtered and kept in small brown bottles for further use.

0.5% Acid-Alcohol (Decolouriser): Conc. HCL – 0.5 ml and 70% Ethanol – 99.5 ml

0.5% Aqueous Potassium Permanganate (Decolouriser): Potassium permanganate solution was prepared by adding 0.5 mg of KMnO4 to 100ml of distilled water and the solution was filtered and kept in dark bottles for further use.

2) Procedure

The prepared slides were kept over the staining rack, then flooded with Auramine Phenol O and left for 15 minutes. Rinsed with water until the stain gets washed away and decolourised with acid/alcohol for 1 minute. The slides were washed thoroughly. 0.5% potassium permanganate solution was added and left for 3 minutes and then rinsed with water and kept for air dry. The stained slides were examined under 40X magnification LED fluorescent microscope. The slides were systematically scanned to cover the whole smear and were graded according to RNTCP guidelines

Table 2: Grading of Smears [7]

	No. of fields to be examined	Grading	Results

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No AFB in 40 fields	40	0	Negative
1-19 AFB/ 40 fields	40	Scanty	Positive
20-199 AFB/ 40 fields	40	1+	Positive
5-50 AFB/ 20 field	20	2+	Positive
>50 AFB/ 8 field	8	3+	Positive

Procedure For Inoculation into Lowenstein Jensen Media: The bottles containing LJ medium were labelled with patient details and the date of inoculation. A loopful of the processed specimen was used to inoculate one slope of Lowenstein Jensen media. All the slopes were incubated at 370C.

Examination of Cultures: The slopes were checked for growth daily for one week and then weekly for eight weeks. The typical colonies of M. tb are rough, tough and buff coloured which appear after first of inoculation. The growth was confirmed by acid fast staining and a rapid TBc ID kit [11]. 0.1 ml of the growth suspension was inoculated into the sample well and incubated for 15 minutes at room temperature.

Figure 2: Lowenstein Jensen Culture Media

CBNAAT:

Procedure [12]

The specimens collected in falcon tubes were mixed with the sample reagent containing NaOH and isopropanol in 1:2 ratio. The mixture was kept at room temperature for 15 minutes with intermittent shaking for 5 seconds. 2ml of the digested specimen was transferred to the CBNAAT cartridge and the lid was closed. The cartridge was loaded into the machine.

Processing of tissue samples

The synovial tissue was cut into small pieces using sterile scissors or forceps in a sterile mortar. 2ml of sterile phosphate buffer (PBS) was added to it and the tissue was grinded to form a homogeneous mixture. line was present, the test was considered invalid. 0.7 ml of the homogenized tissue sample was transferred into a sterile container using sterile transfer pipette. Double volume of sample reagent (1.4 ml) was added to the homogenized tissue using a transfer pipette. The sample was vortexed for at least 10 seconds and incubated for 10 minutes at room temperature. Again, the sample was vortexed for at least 10 seconds and incubated for 5 minutes at room temperature. 2ml of the processed sample was transferred using a transfer pipette to the cartridge. The cartridge was loaded into the machine.

Interpretation of results

The results obtained from CBNAAT assay were: 1) M. TB not detected; 2) M. TB detected; RIF resistance not detected; 3) M. TB detected; RIF resistance detected

Statistical Analysis

The data was analysed with SPSS 21.0 version. To find the significance in categorical data, Chi square was used. Calculations like1) Percentages 2) Proportions and 3) Mean values were taken. Appropriate statistical tests like Chi- Square test, T

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test was used to compare the study parameters. Data were analysed for any statistical significance with a P value <0.05 being considered significant.

Observations and Results

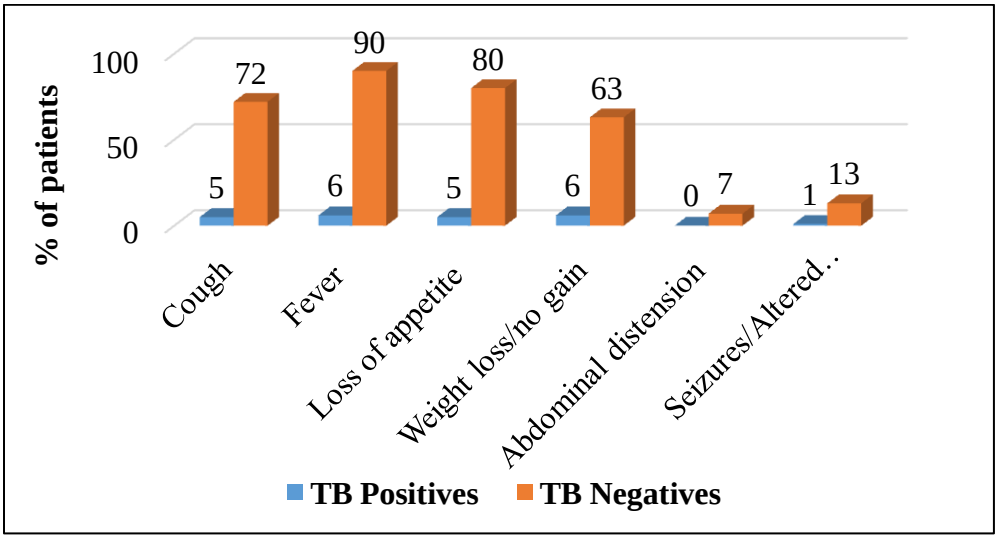
A total 114 children suspected of tuberculosis were included in the study. The overall detection rate of tuberculosis among the study population was 5.3% which was higher in male (7.4%) children less than 5 years of age (4/6=66.66%) and belonging to lower class (10.3%) as shown in table 3.

Table 3: Sociodemographic profile of patients and TB Positivity

Demographic data		TB Positives	TB Negatives	Total cases
Age group (Years)	<1	02 (14.3%)	12 (85.7%)	14 (100%)
	1 to 5	02 (2.7%)	72 (97.3%)	74 (100%)
	6 to 10	01 (4.3%)	22 (95.7%)	23 (100%)
	10 to 12	01 (33.3%)	02 (66.7%)	03 (100%)
	P value	0.045		
Gender	Male	04 (7.4%)	50 (92.6%)	54 (100%)
	Female	02 (3.3%)	58 (96.7%)	60 (100%)
	P value	0.331		
Socio-economic status	Lower class	04 (10.3%)	35 (89.7%)	39 (100%)
	Lower Middle	02 (3.1%)	62 (96.9%)	64 (100%)
	Upper middle	0 (0.0%)	11 (100%)	11 (100%)
	P value	>0.05		

All i.e., 100% of the tuberculosis positive patients had complaints of fever and weight loss/ no weight gain as depicted in figure 3. A statistically significant difference was seen in the cases with weight loss/ no gain (p<0.05).

Figure 3: Distribution of cases according to symptoms and TB positivity



The pulmonary tuberculosis was the predominant form (83%) among the children studied. The contact history was present in 66.7% of the positive cases of tuberculosis. The Mantoux test was positive in 100% of the

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positive cases and all the tuberculosis patients had history of BCG vaccination and showed a BCG scar. Majority of TB positive patients (18.8%) with underweight and 9.4% with severe acute malnutrition.

TB positivity was more i.e., 50% in cases with type of specimen pleural fluid compared to 9.1% and 4.2% of the cases with the type of specimen sputum and gastric aspirate respectively were found to be TB Positive and the different was found be statistically significant with p = 0.014, (Table 4).

Table 4: Distribution of cases according to type of specimen and TB positivity

Type of Specimen	TB Positives	TB Negatives	Total cases	P value
Gastric aspirate	03 (4.2%)	69 (95.8%)	72 (100%)	0.014
Sputum	02 (9.1%)	20 (90.9%)	22 (100%)	
CSF	00 (0.0%)	10 (100%)	10 (100%)	
Induced sputum	00 (0.0%)	04 (100%)	04 (100%)	
Ascitic fluid	00 (0.0%)	4 (100%)	04 (100%)	
Pleural fluid	01 (50%)	1 (50%)	02 (100%)	

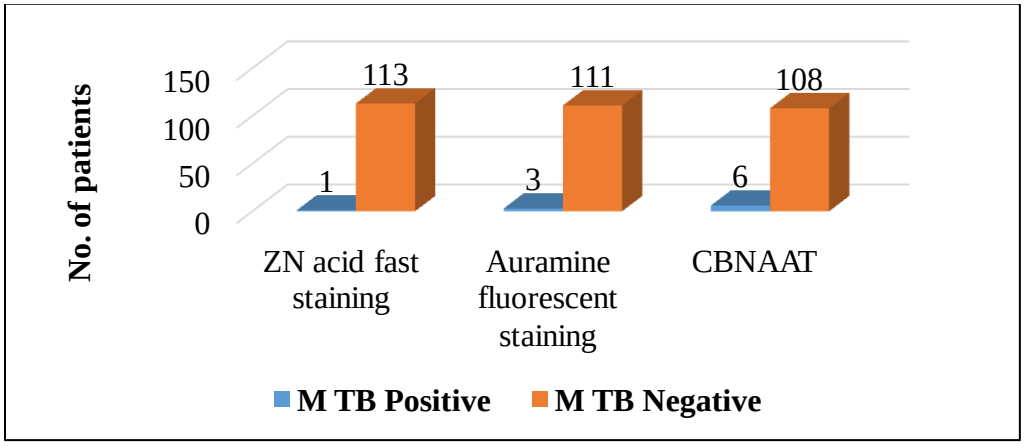
Out of TB positive cases, only 16.7% cases were positive by ZN Acid fast staining. Out of CBNAAT positive cases only 50% of the cases were positive on Auramine Fluorescent Staining as shown in table 5. TB Positivity was 5.1% in cases with pulmonary samples and 6.3% in extra-pulmonary samples and the difference was found to be statistically not significant, (p=0.849).

Table 5: Comparison of CBNAAT with ZN acid fast staining and auramine fluorescent staining

CBNAAT	ZN acid fast staining		Auramine fluorescent staining	
	Positive	Negative	Positive	Negative
M TB Positive	1 (16.7%)	5 (83.3%)	03 (50%)	03 (50%)
M TB Negative	0 (0.0%)	108 (100%)	00 (0.0%)	108 (100%)
P value	0.000		0.000	

Acid fast staining had a sensitivity of 16.6% and specificity of 100%. The Positive and negative predictive value was 100% and 95.6% respectively. Fluorescent staining had a sensitivity of 50% and specificity of 100%. The Positive and negative predictive value was 100% and 97.3% respectively. Out of 6 TB positive cases, only 1 case was tested positive by ZN acid fast staining and 3 cases were tested positive by fluorescent staining, as depicted in figure 4.

Figure 4: Comparison of different diagnostic tests in paediatric TB



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Discussion

In the present study, the detection rate of tuberculosis among children was 5.3%. The worldwide detection rate of paediatric TB increased from 6% (2016) to 10% (2020) in the past five years [13]. Tuberculosis among the paediatric age group mostly results from household contacts of an infectious case [14]. Owing to low clinical suspicion and lack of ideal point of care technologies for the diagnosis, the real data regarding the exact prevalence of tuberculosis in children is not available. Age is a crucial factor in case of paediatric tuberculosis as it determines the risk of transforming infection to disease. The risk increases as the age decreases. In age groups <1 year and 1-5 years the risk of infection is the highest as revealed in current study, the proportion of tuberculosis positive cases was found to be higher among children <5 years of age which is comparable with the study conducted by Shrestha S et al [15]. Male predominance could be appreciated in the detection of tuberculosis in children <5 years of age and older children in current study. Similar reports were observed in many studies [16].

All the tuberculosis positive patients (100%) had complaints of fever which is similar to study conducted by Shrestha S et al [15]. The history of contact with an infectious case was present in 66.4% of the positive cases, this is correlated with the previous studies [17, 18]. However, all the tuberculosis positive children had history of BCG vaccination and showed a BCG scar because of universal BCG vaccination for all children. 18.8% of tuberculosis positive children were underweight and 9.4% of the children were severe acute malnutrition as per WHO classification. In developing countries, Mantoux test remains one of the routine investigations done in suspected cases of paediatric tuberculosis. Although it serves as indirect evidence for diagnosing paediatric tuberculosis, its usage is limited as it may be negative in 10%-25% of active tuberculosis cases [19]. In current study, Mantoux positivity was seen in 100% of the tuberculosis positive cases, this can be because the larger number of tuberculosis positive patients were seen in lesser age groups. This is very high when compared to study done by Vijayasekaran D et al which reported Mantoux positivity of 34.7% in various forms of paediatric tuberculosis [18].

Gastric aspirate showed 4.2% tuberculosis positivity and sputum showed 9.1% positivity which is similar to study conducted by Mukherjee A et al [20]. One patient in present study showed tuberculosis positive for pleural fluid (50%). Similar results are seen in a study conducted by Ruan SY et al [21]. The proportion of pulmonary tuberculosis was 83.4% (n=5) and extra pulmonary tuberculosis was 16.6% (n=1). These findings collaborate with previously published studies [15,16,22].

There was an enormous difference between the two microscopy methods which were used in this study for the diagnosis. The positivity rate was increased by almost 49% by using fluorescent microscopy when compared with acid fast staining method. These findings are comparable with the other studies [23-25]. Thus, fluorescent microscopy is more sensitive in detecting the paucibacillary cases as it can screen more area of smear under higher magnification. This method is also less time consuming when compared with the conventional acid-fast technique. On comparing the staining methods of CBNAAT in detecting positive cases of tuberculosis, all the three methods had almost equal specificities but there was wide difference in sensitivity. The sensitivity of CBNAAT, fluorescent staining method and acid-fast method was found to be 100%, 50% and 16.6% respectively which is comparable with the study done by Agrawal M et al [26]. Researchers from various parts of the country reported varied sensitivities of CBNAAT to be 86%-100% and specificity to be 72%-99% [27]. Thus, CBNAAT could be recommended as a first line of investigation in diagnosing tuberculosis, especially in high prevalence countries.88 On the other hand, CBNAAT gives quicker results and requires minimal biosafety requirements and minimal training for laboratory staff. With culture method, false positives can occur with atypical mycobacterium but with CBNAAT, this could be eliminated. CBNAAT is also useful in retreatment cases for finding out rifampicin resistance.

There are some limitations of the study which includes- 1) It was a single centre study, so it can't be generalised; 2) The study setting having limited number of investigations and 3) Result of the study cannot be applied uniformly across the community as it is purely hospital-based study.

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Conclusion

Among the methods which were used for the diagnosis of paediatric tuberculosis, CBNAAT was advantageous as it could detect more cases which were missed by other conventional methods. CBNAAT gives the result in 2 hours along with rifampicin resistance. We can get the results more rapidly, hence we will come to know status of tuberculosis of the patient and the resistant pattern of organism, so we can decide the treatment according to the resistance pattern. However, it helps in preventing multi drug resistant tuberculosis as the treatment started upon the sensitivity pattern.

Recommendations

CBNAAT testing is one of the quicker ways of diagnosing paediatric tuberculosis and drug resistance pattern. We can get faster results within 2 hours. So, we recommend policy makers to include CBNAAT testing and CBNAAT testing centres at various levels for early diagnosis and treatment. It helps in preventing and eliminating tuberculosis in community and emergence of drug resistance.

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