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Panacea Journal of Medical Sciences

Journal homepage: http://www.pjms.in/

Original Research Article

Utility of cartridge based nucleic acid amplification test in diagnosis of tubercular lymph nodes on fine needle aspiration and its comparison with zeihl-neelsen staining and concentration bleach method

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ARTICLE INFO

Article history:
Received 16-03-2021
Accepted 20-07-2021
Available online 30-04-2022

Keywords:
TB lymph node
ZN stain
Concentration bleach
CBNATT

ABSTRACT

Introduction: Tuberculosis (TB) is one of the oldest disease known to humanity and among the deadliest epidemics and is in the top 10 causes of death worldwide. The incidence of tuberculosis in India has dramatically increased and contributing to it is Human Immunodeficiency Virus (HIV). Various diagnostic methods are available for detection of tubercular bacilli. Only full proof criteria for diagnosis of TB is to demonstrate the bacilli from affected tissue. This study was planned in extra pulmonary tuberculosis cases to evaluate different staining methods for demonstration of mycobacterium on FNAC material.
Aims of study: To evaluate efficacy of Cartridge Based Nucleic Acid Amplification Test (CBNAAT) in the initial cytodiagnosis of Tubercular Lymphadenitis
Material and Methods : 147 clinically suspected cases of extra pulmonary tuberculosis mainly TB lymphadenitis who attended cytology OPD were selected irrespective of age and sex. FNAC was performed. Slides stained for PAP, ZN and concentration bleach. Material remained in hub was sent for CBNAAT. PAP stained slides were observed for various morphological pattern.
Result: Six morphological patterns were observed. Positivity for acid fast bacilli by ZN staining, concentration bleach method and CBNAAT were compared. Detection of acid-fast bacilli by CBNATT was found more as compared to ZN and Concentration bleach method.

1. Introduction

Tuberculosis (TB) is one of the oldest diseases since many decades and one of the deadliest epidemics. One third of world's population infected with mycobacterium tuberculosis and 10 million new cases occur each year. Nearly 1.7 million people die annually due to tuberculosis making it the leading cause of death due to an infectious agent worldwide. [1]

Tuberculosis occurs in all age. The incidence of tuberculosis in developing countries like India has dramatically increased over last few decades. Burden of TB in India as per the Global TB report 2020 is approximately 28 million accounting for about a quarter of the world's TB cases [2] and about 4 million people die annually. This is due to poor standard of living, lack of health resources. Also contributing to it is the Human Immunodeficiency Virus (HIV). Current report suggest India has 2.1million people infected with TB. 87000 people of HIV infected with TB and 12,000 has morbidity due to HIV-TB co-morbidity. [2] In addition to this patients who are defaulter for tubercular regimen developed Multi drug resistance (MDR) bacilli / resistance to Rifampicin which is potent drug in treatment of tuberculosis, leading to public health crisis and health security threat for emerging MDR resistant bacilli. [1]

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https://doi.org/10.18231/j.pjms.2022.012

With the improvement in economic and social condition and use of effective anti –TB therapy (ATT), there is a decline in pulmonary TB cases. However, extrapulmonary TB is now more common and lymphadenopathy is the most common presentation of extrapulmonary TB.[3,4]

Considering the load of tuberculosis infection pulmonary and extrapulmonary, early diagnosis is essential to limit morbidity and mortality. National strategy Programme (NSP), in "India TB report 2020" proposes bold strategies with commensurate resources to rapidly decline TB incidence and mortality in India by 2025, five years ahead of the global End TB targets under Sustainable Development Goals to attain the vision of a TB-free India.[2]

Under this, various diagnostic methods are available for detection of tubercular bacilli. Only full proof criteria for diagnosis of TB is to demonstrate the bacilli from affected tissue. It is well known that acid fast bacilli can be demonstrated in only 25-30% cases showing tubercular granuloma however Zeihl-Neelsen (ZN) staining have low sensitivity for detection of acid-fast bacilli.[5,6]

Improvement in detection rate of AFB has been reported by various techniques, such as concentration bleach technique, by fluorochrome staining methods and now a days by polymerase chain reaction (PCR) and cartridge based nucleic acid amplification test (CBNAAT) methods. These techniques are mainly utilized to demonstrate acid fast bacilli in sputum.[2]

Hence the aim of this study is to evaluate different cytological pattern and to find out efficacy of different staining methods along with cartridge based nucleic acid amplification test for demonstration of extrapulmonary mycobacterium on FNAC material.

2. Materials and Methods

This is a prospective study carried out in department of pathology, Indira Gandhi Govt Medical College, Nagpur in period between 1/1/2018 to 30/10/2019.

2.1. Inclusion criteria

All the cases which were clinically suspected for tubercular lymphadenopathy sent in cytology OPD for FNAC were included in study irrespective of age, sex and site of lymph nodes. Cases were clinically evaluated in cytology OPD for detailed clinical history including history of fever, cough, weight loss, past history of tuberculosis (pulmonary or extrapulmonary), past or recent history of AKT (anti-Koch's treatment) and history of contact to tubercular patients. Detailed investigations were documented on FNAC consent form which included X-ray chest, ESR, Mantoux test.

2.2. Exclusion criteria

Clinically suspected metastasis in lymph node or primary malignancy of lymph nodes, inadequate FNAC material, haemorrhagic smears along with patients who were not willing for FNAC were excluded from study.

147 cases were selected for study. Detailed FNAC procedure was explained to patient and consent was taken on FNAC requisition form. FNAC of lymph nodes were performed with 24-gauge needle. FNAC material was divided into 2 parts. From first part 4 slides were made, 2 wet fixed slide in 95% alcohol for H & E and Papanicolaou (PAP) stain and 2 slides kept dry which were stained for May Grunwald Giemsa (MGG) and ZN stain. Second part of the material in needle hub was divided for modified bleach staining and CBNATT testing. This is routine protocol followed in institute for tubercular infection except modified bleach staining.

2.3. Modified bleach staining method

FNAC aspirate was mixed with 2 ml of 4% sodium hypochlorite. Mixture was incubated for 15 min and equal volume of distilled water was added. Mixture was centrifuged for 15 min on 3000 RPM. Supernatant was discarded and one drop of sediment stained with ZN staining.

2.4. CBNAAT or GeneXpert testing

FNAC material was collected in falcon tube with 1 ml of saline and with proper labelling sample was transferred to TB chest department for CBNAAT testing. CBNAAT performed on GeneXpert machine according to the manufacturer's instructions. Sample reagent was added to FNAC material at a ratio of 2:1 after thorough mixing kept for 10 min at room temperature, then shaken again and kept for 5 min; 2 ml of material was transferred to the test cartridge and inserted into the test platform. Results were documented.

After staining (HE, PAP) slides were evaluated microscopically for cytomorphological patterns. ZN and concentration bleach stained slides were examined under oil immersion and AFB bacilli expressed as the number of organisms seen per 100 or 300 oil immersion field as per guideline (OIF).[7] CBNAAT reports were collected from TB chest department.

2.5. Statistical analysis

All the data was arranged in Microsoft excel sheet. From data age and sex distribution of cases were analysed along with cytomorphological patterns on cytostained smears. After categorising cytology smears in various morphological pattern, ZN stained slide and modified bleach stained slides were evaluated under oil lens, at least 100 fields for AFB bacilli. These results were compared with CBNAAT reports. For statistical analysis MEDCALC software used for calculation of sensitivity, specificity, PPV, NPV, Accuracy along with McNemar test used to find out p

value.

3. Results

Study included total 147 cases of FNAC. Out of 147, 113cases were of cervical lymphadenopathy followed by submandibular lymph nodes, submental lymph nodes. Predominantly cases were in 3rd and 4th decade having female preponderance (78cases out of 147). [Table 1]

On microscopic examination of PAP and H & E stained slides were categorised in six cytomorphological patterns.[5] Category I (25) – reactive lymphadenitis, category II (5)- Reactive with few epithelioid cells, category III (37) - Epithelioid cell granuloma with reactive population, category IV (42)- Epithelioid cell granuloma with caseous necrosis, category V (23)- Caseous Necrosis only, category VI (15)- Degenerative polymorphs. In our study most common morphological pattern was category IV followed by category III and category I. Least common category was category II**.** [Table 2,Figure 2]

All these patterns were evaluated for AFB positivity by ZN staining, Modified Bleach staining and compared with CBNAAT testing. More positivity seen in IV, V and VI category and percentage of detection of more cases seen by CBNAAT i.e.74%. [Table 2]

Among 147 sample 35 samples were positive by ZN staining, concentration bleach and CBNAAT. In addition, 49 cases by modified bleach and 74 cases by CBNAAT was detected. Cases which were negative by CBNAAT were also negative by ZN but two cases were detected by modified bleach method. 38 samples were negative by all three methods. All the cases of category V and VI detected by CBNAAT as compare to ZN and concentration bleach.

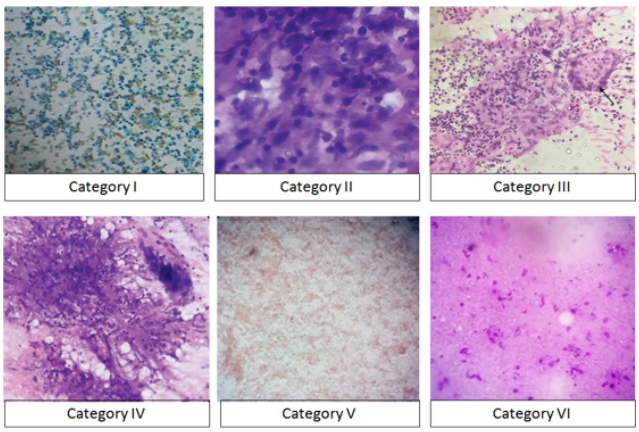

Fig. 1: Cytomorphological patterns on PAP and H& E staining

ZN positivity seen in 23.43 % cases (35/147) (Figure 1). Out of 35 ZN positive cases category V showed more positivity followed by category IV. Not a single case was detected by ZN method in category I and Category II. So, category I was appeared to be purely reactive lymphadenitis after ZN staining.

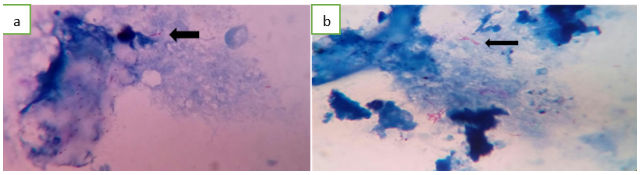

Fig. 2: a: Showing bacilli in zn stain (100X); **b:** Bacilli in modified bleach background (100x)

84 cases (57.03%) were positive by concentration bleach method (Figure 2). Category VI, Category V and Category IV showed higher positivity i.e. 84 %, 83% and 78 % respectively. Category I was negative by concentration bleach method as that of ZN method. 2 cases of category II were detected by concentration bleach method. These cases were not detected by ZN method.

Additional 49 cases were detected by modified concentration bleach as compared to ZN which is significantly high in number.

Out of 147 cases sent for CBNATT, in 109 cases mycobacteria were detected. All cases in reactive category were also negative by CBNATT. However, there were two cases which was positive by concentration bleach method but negative by CBNAAT.

When we applied statistic to compare CBNAAT with ZN staining and concentration bleach method, p value is less than 0.001. Hence CBNAAT is more sensitive in detecting acid fast bacilli i.e. 74.1% than ZN and Modified bleach method.

4. Discussion

According to the WHO's report, the number of people with TB in India is falling and this demonstrates that making serious gains against TB in a short time frame is possible even in the world's largest and most geographically diverse countries. But still not falling nearly fast enough in India, progress is still too slow to meet the targets. Hence in India, under TB eradication programme includes early identification of presumptive TB cases, at the first point of care be it private or public sectors, and prompt diagnosis using highly sensitivity diagnostic tests to provide universal access to quality TB diagnosis including drug resistant TB in the country.

What does it entail?

1. To use high efficiency diagnostic tools for early and accurate diagnosis linked treatment across the country
2. To strengthen surveillance systems including introduction and scale up of next generation sequencing platforms
3. Purchasing services and ensuring notification through laboratories from the private sector and link to laboratory surveillance

Table 1: Age distribution amongst male and females

Age of patients in years	Male	Female	No. of Cases 147 cases	Percentage
1 - 10	3	3	6	4.68%
11 – 20	10	14	24	18.75%
21 - 30	24	28	52	35.5%
31 - 40	17	25	42	28.5%
41 - 50	7	4	11	8.59%
51 – 60	8	3	11	4.68%
>60	0	1	1	0.74%
Total	69	78	147	

Table 2: Morphological patterns with positivity (Figure 1)

Category	Total cases	ZN positivity	Bleach method positivity	CBNAAT
I. Reactive lymphadenitis	25	0	0	0
II. Reactive with few epithelioid cells	5	0	02	02
III. Epithelioid cell granuloma with reactive population	37	03	13	30
IV. Epithelioid cell granuloma with caseous necrosis	42	12	33	39
V. caseous necrosis only	23	12	22	23
VI. Degenerative polymorphs	15	08	14	15
Total	147	35(23.8%)	84(57.14%)	109(74.14%)

Table 3: Cytomorphological pattern compared with other studies [5,8–10]

Pattern	Present study	Hemlata et al	Chandrashekharn et al	Priyanka chand et al	Khajuria R et al
I. Reactive lymphadenitis	17%	32%	38	19%	
II. Reactive with few epithelioid cells	3.4%	17%	7%		
III. Epithelioid cell granuloma with reactive population	25%	25%	24%	28%	41%
IV. Epithelioid cell granuloma with caseous necrosis	29%	25%	15%	21%	30%
V. caseous necrosis only	16%	17%	7%	15%	43%
VI. Degenerative polymorphs	10%		14%	17%	

Table 4: omparison of CBNAAT with ZN staining and concentration bleach

n=147	ZN staining			Concentration bleach method		
CBNAAT	positive	negative		Positive	Negative	
Positive	35	74	109	82	27	109
Negative	0	38	38	2	36	38
	35	112	147	84	63	147

4. To promote and foster research for new diagnostic tools
5. To build capacity for diagnosis of LTBI

Hence the detection of acid fast bacilli is considered as the evidence of the infection. Thus, the laboratory plays a critical role in the diagnosis of TB. In developing countries, microscopy of the specimen is the fastest, cheapest, and most reliable method for the detection of AFB. The present study showed that out of 147 lymph nodes aspiration, CBNAAT was positive in 109 patients and 84 patients were positive with concentration bleach method, 35 Ziehl-Neelsen's stain. Sensitivity of CBNAAT in detecting TB bacilli in sputum was well documented [11–13] however in current study CBNAAT is found to be more sensitive method in demonstrating tubercular bacilli in lymph nodes aspiration as compared to ZN and concentration bleach method. CBNAAT improves the detection rate, especially in patients with low density of bacilli that are likely to be missed on Ziehl-Neelsen's and concentration bleach method stain. Only two cases which were diagnosed on concentration bleach method were missed by CBNAAT. As we distributed the sample for various diagnostic methods hence there are chances of either no material or non-

Table 5: statistical analysis of CBNAAT with ZN staining

n=147	CBNAAT		Total
ZN staining	**Positive**	**Negative**	
Positive	35	0	35
Negative	74	38	112
Total	109	38	147
Parameter	**Estimate**	**Lower - Upper 95% CIs**	
Sensitivity	32.11%	(24.08, 41.36[1])	
Specificity	100%	(90.82, 100[1])	
Positive Predictive Value	100%	(90.11, 100[1])	
Negative Predictive Value	33.93%	(25.82, 43.1[1])	
Diagnostic Accuracy	49.66%	(41.69, 57.65[1])	
Cohen's kappa	0.1965	(0.1003 - 0.2927)	
Chi square McNemar		73.01	
P value		<0.001	

Table 6: Statistical analysis of CBNAAT with concentration bleach staining

n=147	CBNAAT		Total
Concentration bleach method	**Positive**	**Negative**	
Positive	82	2	84
Negative	27	36	63
Total	109	38	147
Parameter	**Estimate**	**Lower - Upper 95% CIs**	
Sensitivity	75.23%	(66.36, 82.38[1])	
Specificity	94.74%	(82.71, 98.54[1])	
Positive Predictive Value	97.62%	(91.73, 99.34[1])	
Negative Predictive Value	57.14%	(44.86, 68.6[1])	
Diagnostic Accuracy	80.27%	(73.1, 85.9[1])	
Diagnostic Odds	54.67	(12.33 - 242.3)	
Cohen's kappa	0.5762	(0.4257 - 0.7267)	
Chi square McNemar		21.55	
P value		<0.001	

representative material had been sent for CBNAAT testing. CBNAAT is found to be more superior in detecting TB bacilli as compared to Ziehl-Neelsen's and concentration bleach method in smears with diagnostic cytomorphological features of tuberculosis like acute inflammatory exudates or only epitheloid cell granuloma. One can find Rifampicin resistant bacilli by using CBNATT. Genxpert is financially expensive but having such instrument in district level or bigger institute help in detecting early tubercular cases along with Rifampicin resistance , where already load of infection is high. CBNAAT is giving reports in one or two days while gold standard culture method takes three weeks for result. So definitely CBNAAT is useful in terms of time, detecting MDR TB cases and ultimately cost effective. The potential benefits of automated screening for tubercle bacilli are: rapid, acute, inexpensive; the ability to screen large number of people; increased resources to monitor patients; and reduction in health risk to staff.

5. Conclusion

CBNAAT is more sensitive and specific in detection of extra pulmonary TB and pauci bacillary cases in FNAC material along with of rifampicin resistance. In national tuberculosis elimination programme central government also recommended CBNAAT testing for early detection of tubercular bacilli[2] Therefore, CBNAAT should be used for early diagnosis not to miss the pauci bacillary cases as it increases rapid case detection by 10-20% and plays an important role in effective patient management that can help in reducing incidence and mortality due to tuberculosis and ultimately achieving the goal "TB free India".

6. Acknowledgment

We acknowledge TB and Chest department for CBNAAT testing

7. Source of Funding

None.

8. Interest of Conflicts

None.

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Cite this article: Dhote AR, Gaikwad A, Datar S, Kowe B. Utility of cartridge based nucleic acid amplification test in diagnosis of tubercular lymph nodes on fine needle aspiration and its comparison with zeihl-neelsen staining and concentration bleach method. *Panacea J Med Sci* 2022;12(1):61-66.