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Utility Of PCR In The Diagnosis Of Tubercular Lymphadenitis On Fine Needle Aspirates.

Swapna Mallure*.

Associate Professor, Department Of Microbiology, Sri Lalithambigai Medical College And Hospital, Adayalampattu, Maduravoyal, Chennai, Tamil Nadu, India.

ABSTRACT

Extra-pulmonary tuberculosis is a diagnostic and therapeutic challenge. Diagnosing tubercular lymphadenitis includes identification of epithelioid granulomas, positive Acid Fast Bacilli detected with Ziehl Neelsen's stain. The disadvantage is the time taken for formation of granulomas. With the advent of RT-PCR, the diagnosis of tubercular lymphadenitis can be made with ease.o utilize PCR and mycobacterial culture on samples obtained from Fine needle aspiration of lymph nodes in order to aid in early diagnosis of extra-pulmonary tuberculosis. This Observational descriptive prospective study was done in the year 2023 , Department Of Microbiology, Sri Lalithambigai Medical College And Hospital Adayalampattu, Maduravoyal, Chennai, Tamil Nadu India .Patients with lymphadenopathy >1 cm, above the age of 2 years were included ; FNA was performed, material was used for routine cytological studies (PAP, MGG and Ziehl Neelsen-stained slides), PCR and culture (wherever possible); Comparative values of PCR and culture were utilized for statistical analysis. Out of 80 cases studied, M. Tuberculosis by PCR was detected on 36 cases (45%); PCR and culture demonstrated MTB in 42.55% of the cases. 9 cases (11.25%) of reactive lymphadenitis were found to be of tubercular etiology on PCR analysis. The Chi-squared test was highly significant. PCR has been found to have a sensitivity, specificity, PPV and NPV of 100%, 92.59%, 90.91% and 100%, respectively. Positive RT-PCR was more common in cases with chronic granulomatous inflammation when compared against reactive lymphoid hyperplasia with a chronic inflammatory infiltrate. From this study, we conclude that RT-PCR is a useful ancillary technique to conclusively identify Mycobacterium tuberculosis in samples obtained from fine needle aspiration of lymph nodes.

Keywords: Tubercular Lymphadenitis, PCR, Fine-Needle Aspiration Cytology, Lymphadenopathy.

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Corresponding author

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INTRODUCTION

Tuberculosis is caused by the bacillus *Mycobacterium tuberculosis*, which typically affects the lungs but can also affect other sites resulting in extrapulmonary tuberculosis. Lymph nodes are the most affected extrapulmonary site. Early diagnosis and initiation of first-line treatment can prevent transmission and curtail the infection. Globally an estimated number of 10 million people were infected by tuberculosis and India has the highest burden of tuberculosis accounting for one-fifth of the global incidence [1]. Tuberculosis has continued to pose many challenges to the public health system. Extrapulmonary tuberculosis, on the other hand, continues to remain a diagnostic. Granulomatous lymphadenopathy has an exhaustive list of differential diagnosis comprising of tuberculosis, sarcoidosis, fungal infections and other inflammatory conditions. An added complexity is a high clinical suspicion of extrapulmonary tuberculosis but lack of bacteriological proof on conventional microscopy [2, 3].]The standard protocol for tubercular lymphadenitis on cytology includes identification of epithelioid granulomas with giant cells, caseation necrosis and a positive Acid Fast Bacilli detected with Ziehl Neelson's stain. The disadvantage with the above-mentioned procedure is the time taken for formation of granulomas which is a response to cell mediated immunity and can take up to 100 days and this accompanied with the poor sensitivity of Ziehl Neelson's stain can result in an advertent delay in diagnosis. Fluorescent stains have been proven to have a superior sensitivity when compared to standard Ziehl Neelson's stain. With the advent of RT-PCR, the diagnosis of *M.tuberculosis* can be made with ease [4]. The gold standard however continues to remain the method of culture which generally requires 10-100 live bacilli per mL but bears the disadvantage of taking up to eight weeks for detection. PCR, on the other hand, is a rapid method to detect mycobacterial DNA, even in small quantities or non-viable clinical samples [5]. Over the past few years, fine needle aspiration cytology has proved to be an important tool in the diagnosis of extra-pulmonary tuberculosis. The chief difficulty in this method is the low quantity of sample obtained which is frequently only utilized to prepare smears even for Ziehl-Neelson's and fluorescent staining. Combining the methods of fine needle aspiration cytology and PCR would result in an effective step for the early diagnosis of tuberculosis [5].

This study was undertaken to assess the utility of RT-PCR in the diagnosis of tubercular lymphadenitis.

MATERIALS AND METHODS

This Observational descriptive prospective study was done in the year 2023, Department Of Microbiology, Sri Lalithambigai Medical College And Hospital Adayalampattu, Maduravoyal, Chennai, Tamil Nadu India A total of 80 patients were included in the study.

Inclusion criteria

- Patients with lymphadenopathy more than 1 cm referred for fine needle aspiration cytology
- Both sexes and patients above the age of 2 years

Exclusion criteria

- Clinically suspected malignancy in the lymph nodes(metastasis/lymphoma)
- Cytologically diagnosed malignancies
- Children below 2 years of age (To exclude BCG lymphadenopathy)
- Previously treated cases of tuberculosis

Methodology

The protocol and methodology were explained to the patient or guardian (in case of a minor) and a written informed consent was obtained from each of them. A focused clinical and physical examination was conducted. Constitutional symptoms like fever, loss of weight, cough with expectoration or malaise were noted. Clinical examination of the site, size and consistency of the lymph node was also documented. Fine needle aspiration was performed under aseptic precautions using a 23 gauge needle and 10 mL disposable syringe. Multiple passes were performed. The material obtained was divided into various parts. One part was used to prepare a wet fixed smear for Papanicolaou staining. Two parts were used in the

preparation of air dried smears for MGG and Ziehl-Neelson's staining. The hub of the needle was flushed with about 0.5- 1.0 mL of normal saline and the material was collected in a labelled, sterile vial for PCR. Any remaining material was utilized for culture by BACTEC MGIT method. The specimens were immediately transported for their respective methods of processing. Hence, culture was performed only in cases with sufficient material for the same. Cytological interpretation was done on Pap, MGG and ZN stained slides. Interpretation was based on the cellularity, presence of epithelioid cell granulomas, Langhans type of giant cells and presence or absence of caseation necrosis. For culture, it was inoculated into MGIT broth as per the manufacturer's instructions and incubated at 37 degrees Celsius for a maximum of 45 days. The automated MGIT equipment took a reading on alternate days in the first week and thereafter, once a week. If the reading was positive an AFB smear was made from the growth and a rapid card test was performed to differentiate between *M. tuberculosis* and non-tubercular mycobacterium. For the procedure of RT-PCR, 0.1-0.2 mL of the aspirate was mixed in 2mL of buffer in a sterile centrifuge tube which was provided in the kit. The sample was vortexed for 10 minutes and a repeat vortex was performed for 5 minutes. The sample was inoculated into MTB-Rif cartridge and loaded as per the manufacturer's instructions. The PCR products were identified by agarose gel electrophoresis and combined ethidium bromide staining followed by Southern blot hybridization to an α-^{32}P-labeled 306-bp PCR product from the genomic DNA of *M. Tuberculosis* $H_{37}Ra$ with the BW6 and BW7 primers directed against the IS1081 sequence. The reading was taken as follows – MTB- not detected/detected (very low/low/medium/high) and Rifampicin resistance, if any, was noted.

Statistical Analysis

Statistical analysis was performed using JASP 0.11.1 software to calculate sensitivity, specificity, positive predictive value and negative predictive value for the various modalities in the study. The PCR and culture results (wherever available) were tabulated in a 2x2 contingency table. Samples positive on both PCR and culture were considered as true positive while samples negative on both PCR and culture were considered as true negative. PCR positive and culture negative samples were taken as false positive and PCR negative with a positive culture was considered as a false negative. The culture result (wherever available) will be considered as the gold standard. Chi- squared test was used to assess the agreement and a p value of <0.05 was considered as statistically significant.

RESULTS

A total of eighty patients were included in this study. Females comprised 55% of the study population while males comprised 45% with a M:F ratio of 0.8:1. The age groups ranged from 10-81 years of age and the most common age group was the fourth decade accounting for 21.25% of the study population (Table 1). Cervical lymphadenopathy amounted to 67.5% and was thus, the most common site of lymphadenopathy in our study (Table 2). Granulomatous lymphadenitis (38%) was the most common cytological diagnosis followed by reactive lymphadenitis (29%), tuberculous lymphadenitis (26%),necrotizing lymphadenitis (6%) and non-diagnostic (1%). Ziehl-Neelson's stain was performed in all the cases (Table 3). Acid fast bacilli by this staining method were demonstrable in around 24% of these cases and negative in the remaining cases. PCR was able to detect *M. tuberculosis* in 45% (36 cases) out of the 80 cases studied. The quantification of MTB was predominantly a low detection (20 cases – 55%).Nine cases did not reveal typical cytological features of tuberculosis, wherein, there was no evidence of epithelioid granulomas, necrosis or multinucleated giant cells in the smears and their corresponding Ziehl-Neelson's stained slides were also negative. All of the nine cases were diagnosed as reactive hyperplasia of the lymph node on cytological analysis. The PCR analysis of these aspirates was able to detect *M. tuberculosis*. Sixteen cases were cytologically classified as granulomatous lymphadenitis with a negative Ziehl-Neelson's staining and PCR was unable to identify MTB in these aspirates. Cytology alone had a sensitivity of 52.94%, specificity of 97.14%, positive predictive value of 94.74% and negative predictive value of 68% (Table 5).PCR could detect sixteen (20%) additional cases which were missed with ZN staining. BACTEC MGIT was performed in 59% of the cases. Culture was able to pick up *M. tuberculosis* in 25% of the cases. Two cases (2.5%) of a false positive result on PCR were noted, however, there were no false negative cases.A Pearson's two-tailed test of linear correlation between Ziehl-Neelson's stain for acid fast bacilli and RT-PCR established a highly significant relationship with a p value<0.001. This demonstrates that PCR is an efficacious modality for the detection of *M. tuberculosis* when compared against the conventional ZN staining method (Table 4).A similar statistical correlation between PCR and BACTEC MGIT culture for MTB also showed a highly significant relationship ($p <0.001$) (Table 4).In the present study, PCR has a sensitivity of 100%, specificity of 92.59%. positive predictive

value of 90.91%, negative predictive value of 100% and an accuracy of 95.74% (Table 5)

Table 1: Age wise distribution of cases.

Age	Frequency	Percentage (%)
2-10	1	1.25
11-20	20	25
21-30	13	16.25
31-40	17	21.25
41-50	10	12.5
51-60	9	11.25
61-70	7	8.75
71-80	2	2.5
81-90	1	1.25

Table 2: Distribution of cases based on the site of lymphadenopathy.

Site	Frequency
Abdominal	2 (2.5%)
Axillary	7 (8.75%)
Cervical	54 (67.5%)
Others	1 (1.25%)
Inguinal	2 (2.5%)
Submandibular	6 (7.5%)
Submental	1 (1.25%)
Supraclavicular	7 (8.75%)

Table 3: Cytological interpretation.

Cytological impression	Frequency
Granulomatous lymphadenitis	30 (38%)
Necrotizing lymphadenitis	5 (6%)
Reactive lymphadenitis	23 (29%)
Tuberculous lymphadenitis	21 (26%)
Non diagnostic	1 (1%)

Table 4: Comparison among ZN staining, PCR and BACTEC MGIT.

	ZN	PCR	BACTEC MGIT
Detected	19 (24%)	36 (45%)	20 (25%)
Not detected	61 (76%)	44 (55%)	27 (34%)
Not performed	Nil	Nil	33 (41%)

Table 5: Statistical parameters for PCR.

Statistical parameter	Value	95% CI
Sensitivity	100.00%	83.16% to 100.00%
Specificity	92.59%	75.71% to 99.09%
Positive Predictive Value	90.91%	72.49% to 97.43%
Negative Predictive Value	100.00%	
Accuracy	95.74%	85.46% to 99.48%

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Table 6: Statistical parameters for cytological interpretation

Statistical parameter	Value	95% CI
Sensitivity	52.94%	35.13% to 70.22%
Specificity	97.14%	85.08% to 99.93%
Positive Predictive Value	94.74%	71.76% to 99.22%
Negative Predictive Value	68.00%	59.69% to 75.30%
Accuracy	75.36%	63.51% to 84.95%

Figure 1: Caseous necrosis with scattered lymphocytes, Pap,400x.

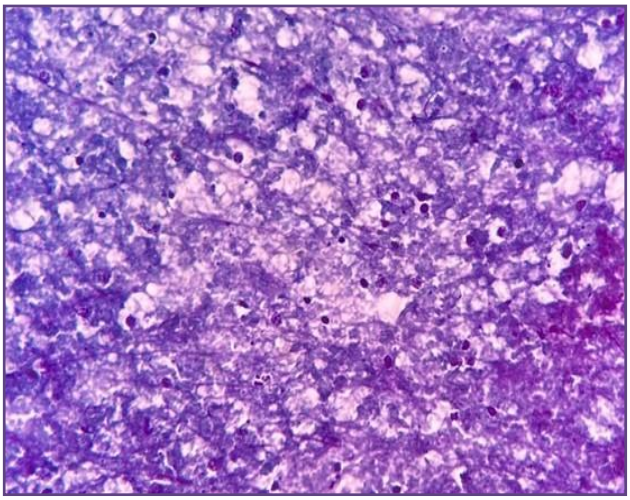
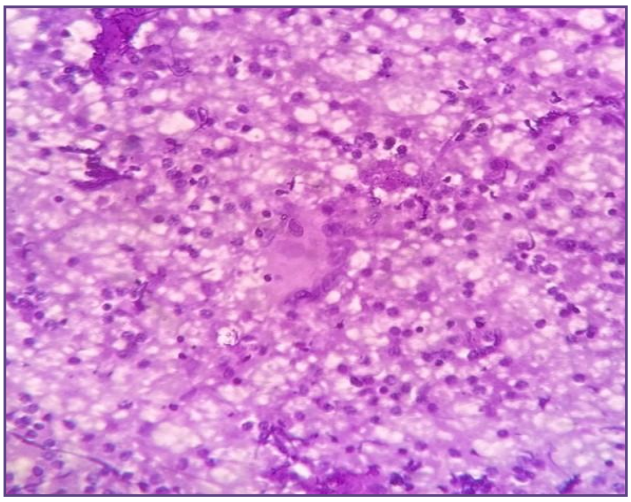


Fig. 2 : Cytological smear with a Langhans type of giant celladmixed with lymphocytes and occasional histiocytes.



DISCUSSION

Until recently, a definitive diagnosis of tubercular lymphadenitis was possible only with a mycobacterial culture which resulted in a delay in diagnosis. This ledto the advent of using PCR to initiate early diagnosis and treatment in tubercular lymphadenitis.[6] The clinician considers clinical history with radiological and pathological findings in order to initiate anti-tubercular therapy. The most common technique employed in day- to-day practice is fine needle aspiration cytology due to its ease of performance with a high reliability. Excision biopsy for histopathological examination is not a treatment of choice due to the high risk of long-term drainage at the surgical site [6]. The cytological appearances of tuberculous lymphadenitis are myriad and comprise epithelioid granulomas,

multinucleated giant cells (Figure-2) or could just exhibit extensive caseation necrosis (Figure-1). This spectrum of features is a result of various factors such as the immune status of the host, socio-economic class and nutritional level [7]. In the present study, most of the cases were between 31-40 years of age. Various other studies have reported different age groups ranging from 18 months to 85 years [4]. Pahwa et al and Kaur et al noted a predominance of patients in thethird decade while Patwardhan et al obtained a median ageof 35 years in their study [4, 13, 14]. A female preponderance was observed in this study with a similar finding in studies by Gupta et al and Patwardhan et al [7, 14]. This gender predilection towards females could be reflective of hormonal, socio-economic and environmental factors. Fundamentally, a difference in the immune systems between males and females is also known. In a developing country like India, even the poor nutritional status is an important predictor of the immune status of the host [8]. On cytomorphology, we noted granulomatous lymphadenitis was the most common category, this is similar to the observations by Kaur et al, whereas Gupta et al noted a preponderance of reactive hyperplasia of the lymph node on cytomorphological analysis [4, 7]. For cytological smears alone, we obtained a sensitivity of 52.94% and specificity of 97.14%; these values are similar to those obtained by Gupta et al, their sensitivity was 53.6% with a specificity of 79.3% [7]. ZN staining is known to have a low positivity, similarly,it was positive in 24% of the cases in the present study.In a study by Gupta et al [4], 29.6% of the cases exhibited a positive ZN staining, while Kaur et al noted a positivity in 20% of their cases [4]. The low rate of positivity for ZN staining could be due to the low volume of sample on the smear, as the bacilli cannot be detected in such cases. A minimum of 10,000-1,00,000 bacilli/mL are required to bedetected on ZN staining [8-10]. PCR could detect an additional nine cases which were cytologically interpreted as reactive lymphoid hyperplasia.A minimum bacillary load of 1000 bacilli/mL is the aspirateis sufficient to be identified by PCR [11]. The probable cause of this false negative on cytology could be due to a mycobacterial load lower than the minimal value or early stage of the disease wherein the granulomatous formation was not yet fully developed [7]. One case was cytologically classified as tuberculous lymphadenitis with a positive ZN staining, however, its corresponding PCR was negative for MTB and culture was not performed in this case. The probable reason for this could be a sampling error or a mutated strain of *M. tuberculosis*. PCR and culture showed maximum positivity in cases which were cytomorphologically categorized as tuberculous lymphadenitis with features of epithelioid granulomas and extensive caseation necrosis, similar to the results of Gupta et al [7] and Pahwa et al [13] on the other hand, few studies have shown a higher rate of PCR positivity on aspirates with features of only necrosis [12]. For PCR, we obtained a sensitivity of 100% and specificity of 92.59%. Gupta et al [7] obtained a sensitivity of 79.5% and specificity of 88.2. The Pearson's chi-squared test was highly significant for PCR and culture, indicating a strong positive relationship between these two methods. These findings are consistentwith other studies by Gupta et al [13].

CONCLUSION

PCR on fine needle aspirates of extrapulmonary lymphadenopathy has proved to be an effective molecular modality in clinically suspected cases of tubercular lymphadenopathy. Routine implementation of RT-PCR in the evaluation of such cases would alleviate the need for invasive procedures, such as, biopsy. A combined team of doctors involving pathologists, microbiologists and clinical specialists are required in the diagnostic approach for suspected cases of tubercular lymphadenitis.

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