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IS TOXOPLASMA A DIFFERENTIAL IN SUPERFICIAL LYMPH NODE ASPIRATES SHOWING GRANULOMATOUS PATHOLOGY?

Pathology

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ABSTRACT

Background: Peripheral lymphadenopathy is frequently due to a local or systemic, benign, self-limited, infectious disease or malignancy. Cervical lymph nodes are involved more often than the other lymphatic regions. In India tuberculosis (TB) is a major benign cause in adults and children. Some studies have been conducted to assess sero prevalence of toxoplasmosis, another infectious disease of significance. The clinical overlap of spectrum between various diseases like early TB, sarcoidosis and toxoplasmosis has led us to investigate in this grey zone. **Methodology:** FNAC from enlarged cervical nodes of 100 patients was done. DNA extraction from the aspirate and Antigen –antibody reaction (ELISA) on serum samples was done on 49 patients who showed granulomatous pathology on cytology smears, out of 100 samples collected. **Results:** There were no IgM antibodies detected in any serum sample. IgG was detected in 7 cases. PCR on the lymph node aspirates did not show any presence of B1 gene of Toxoplasma. **Conclusion:** We recommend that a differential of Toxoplasma be kept as a possible diagnosis when Zeihl Neelsen does not detect AFB on the granulomatous lymphadenopathies. Serologic confirmation in all suspected cases of this self-limited condition should be done, for which no treatment is necessary.

KEYWORDS

FNAC, PCR, Toxoplasmosis, Tuberculosis

INTRODUCTION:

Peripheral lymph nodes, located deep in the subcutaneous tissue, clear antigens from the extracellular fluid. Generally, a normal sized lymph node is less than one cm in diameter. Peripheral lymphadenopathy is frequently due to a local or systemic, benign, self-limited, infectious disease or less frequently a malignancy. Seventy-five percent of all cases of enlarged lymph nodes are localized, with more than 50% being seen in the head and neck area. Cervical lymph nodes are involved more often than the other lymphatic regions. Based on different geographical areas, the etiology varies. For example, in tropical areas, tuberculosis (TB) is a major benign cause in adults and children. In India some studies have been conducted to assess sero prevalence of toxoplasmosis, another infectious disease of significance. One such study has shown a prevalence of 24.3%, with lower rates in northern India and higher in southern India [1] while another study showed a prevalence of 7.7% in adult women [2]. Complete history taking and physical examination are mandatory for diagnosis; however, laboratory tests, imaging diagnostic methods, and tissue samplings are the next steps [3].

The clinical overlap of spectrum between various diseases like early TB, sarcoidosis and toxoplasmosis has led us to investigate in this grey zone. Toxoplasmosis is usually seen in patients associated with a rural background or those having contact with either animals or soil. Many cases have been detected on autopsy from causes of death remaining unknown. Malaise or listlessness along with enlarged lymph nodes especially at the angle of jaw, pre and post-auricular regions and behind the sternocleidomastoid are commonly seen.

Since fine needle aspiration cytology (FNAC) is routinely done in the department of Pathology and the superficial lymph nodes are easily accessible, we intend to investigate and diagnose those cases that are reported as 'granulomatous lymphadenitis' without being confirmed as tuberculosis which is the commonest cause of this clinical presentation. It is possible that many cases of toxoplasmic lymphadenitis are being diagnosed as granulomatous lymphadenitis. This may lead to unnecessary and potentially toxic treatment for tuberculosis. This study hence aims to diagnose the patients who have toxoplasmosis so that the clinicians may treat them for toxoplasmosis instead of exposing them to the anti tuberculosis drugs unnecessarily.

The organism *Toxoplasma gondii* is a common cause of lymphadenopathy in children and adults. Studies have shown that infection can be suspected from histopathology of the lymph node, even though the primary diagnosis rests on the demonstration of specific antibodies in the serum. Several studies have correlated histological features in the lymph node with serology or polymerase chain reaction for the toxoplasma genome in the lymph nodes [4-7].

The histopathological triad of florid reactive follicular hyperplasia, clusters of epithelioid histiocytes, and focal sinusoidal distension by monocytoid B cells has been considered to be diagnostic of toxoplasmic lymphadenitis [8].The occurrence of epithelioid cells within germinal centres is thought to be a specific feature of toxoplasmosis.

Polymerase chain reaction as a reference standard for acute lymphadenitis due to toxoplasmosis has the problem of being oversensitive because it may pick up past infections. IgM ELISA is usually positive in all patients within two weeks of infection and stays positive for the first three months [9] but may also be problematic on occasion because high antibody titres can persist for long periods [10].We will try to minimise this by limiting our subject selection to lymphadenopathy of less than six months duration.

MATERIAL AND METHODS:

Study Setting: Tertiary care centre based study
Study Design: Prospective observational study
Sample Size: 100 over a period of one year
Participant Selection: Patients with superficial lymphadenopathy of more than four (4) weeks duration

Exclusion Criteria:

1) Acute lymphadenitis (<4 weeks duration) due to any obvious infective focus or high grade fever
2) Lymphadenopathy associated with a known malignancy
3) Patients not willing to participate

Steps:

After obtaining informed consent according to the institutional guidelines, study patients were subjected to FNAC from palpable superficial lymph nodes using a 21-gauge needle under sterile conditions and aspirate split in two different portions. One part were used for preparation of smears for hematoxylin and eosin/Giemsa staining for cytological analysis. The material in the hub of the needle was pushed through with 1ml of normal saline into a sterile container

and the mixture then stored at -20°C until used for DNA extraction for Toxoplasmosis. Once the possibility of granulomatous lymphadenitis was diagnosed on cytology, baseline investigations to rule out Tuberculosis were also carried out, e.g. Ziehl-Neelsen staining. The duration of clinical symptoms and the location of affected lymph nodes was recorded on a questionnaire.

5 ml of peripheral venous blood was collected at the time of FNAC for ELISA IgG and IgM for toxoplasma. Serum was separated immediately and stored at −20°C.

Investigations: FNAC, Antigen –antibody reaction (ELISA)
Statistics: Student's *t* test or Kruskall Wallis test as appropriate for quantitative variables. The diagnostic efficacy (sensitivity, specificity, predictive values, and likelihood ratios) of variables with significant differences between the groups will be calculated.

1) Patients presenting with cervical lymphadenitis and had come for FNAC were part of the study population.
2) Ruling out neoplastic causes of lymphadenopathy by careful and through history taking and physical examination was done.
3) The ones with cervical lymphadenopathy and with no obvious malignant were identified.

Case definition:
- patients of any age and either sex
- presenting with non- neoplastic, non-suppurative cervical lymphadenopathy
- no obvious primary lesion

Exclusion criteria:
- Obvious neoplastic lymphadenopathy i.e. Lymphoma, history of primary lesion.
- Obvious infective aetiology.
- Patients not willing to give consent for drawing of blood sample.

4) FNAC done on those patients as a routine.
5) Slides were prepared, stained and studied and reported as part of the routine.
6) Cervical lymph node aspirates of those patients were also taken in Eppendorf containing 2ml of normal saline for DNA extraction.
7) Cervical node aspirate samples in normal saline were then stored at -20^0 C.
8) 3 ml of venous blood samples of the identified patients taken in red-topped vials.
9) Blood samples centrifuged at 1200 rpm for 5 minutes after clot formation and serum separated.
10) Serum samples were then stored at -20°C.
11) The cases with granulomatous pathology were identified.
12) Ziehl Neelsen stain done on granulomatous lymph aspirates to rule out presence of acid fast bacilli (Mycobacterium tuberculosis)
13) IgG and IgM antibodies for Toxoplasma on serum samples by ELISA Principle: Purified Toxoplasma gondii antigen is coated on the surface of microwells. Diluted patient serum is added to the wells, and the Toxoplasma gondii IgG-specific antibody, if present, binds to the antigen. All unbound materials are washed away. HRP-conjugate is added, which binds to the antibody-antigen complex. Excess HRP-conjugate is washed off and a solution of TMB Reagent is added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of IgG-specific antibody in the sample. The results are read by a microwell reader compared in a parallel manner with calibrator and controls.

14) DNA extraction from the lymph node aspirate samples was done. DNA Extraction: DNA was extracted from 200 µl cervical lymph node aspirate samples by using PureLink genomic DNA kit (Invitrogen by life technologies, Carlsbad, CA USA) according to manufacturer's instructions.

15) Nested Polymerase chain reaction was conducted for amplification of B1 gene of toxoplasma.

Nested PCR:
Nested PCR was done of B1 gene of T. gondii (Habibi et al., 2012). Positive control was provided by AIIMS New Delhi on personal request. Primer sets used were

Round 1 Forward 5'-TGTTCTGTCCTATCGCAACG-3'
Round 1 Reverse 5'-ACGGATGCAGTTCCTTTCTG-3'
Round 2 Forward 5'-TCTTCCCAGACGTGGATTTC-3'
Round 2 Reverse 5'-CTCGACAATACGCTGCTTGA-3'

Amplified products were 580bp and 531bp in first and second round amplification respectively. Reaction conditions were same for both round of amplification which was as under.

Initial denaturation at 94 degree C for 5 min, followed by 35 cycles of denaturation at 94 degree C for 30 sec., annealing at 48 degree C for 45 sec., extension at 72 degree C for 45 sec. and a final extension at 72 degree C for 7 min. Amplified products were visualised by using 1.5% agarose gel with ethidium bromide. Ladder of 100bp was used as a reference marker.

16) Gel electrophoresis was conducted to detect the DNA against a positive control.

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, you can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths).

17)Results correlated and interpreted.

RESULTS:
Diagnosis on cytology:
Table 1: Study sample hence consisted of 49 patients who showed granulomatous pathology, out of 100 samples collected.

Table 2: Demography of patients with granulomatous lesion: There were 20 males and 29 females

Table 3: Ziehl Neelsen positivity in granulomatous lymph node aspirates: Ziehl- Neelsen : positive for Acid fast bacilli in 6 of 49 cases.

Table 4: ELISA results: Toxoplasma IgM: there were no IgM antibodies detected in any serum sample.

Toxoplasma IgG: Detected in 7 out of 49 cases Diagnosis on serum samples of patients with granulomatous lymph nodes that were Ziehl-Neelsen negative , showed no rise in IgG and IgM anti toxoplasma antibodies.

Polymerase chain reaction: **Figures 1 and 2** The PCR on the lymph node aspirates did not show any presence of B1 gene of Toxoplasma.

DISCUSSION:
Toxoplasmosis is an infection caused by *Toxoplasma gondii*, a protozoan. The primary host are cats while many mammals serve as its intermediary hosts. Toxoplasma can be transmitted by the feline faeces contaminated soil or food. Most studies have been done on women of child bearing age as it causes congenital defects in fetus. The first national serological prevalence of *Toxoplasma gondii* in India was carried on 23,094 serum samples with *T. gondii* IgG and IgM antibodies by a solid-phase immune capture ELISA. IgG were found in 24.3% while IgM antibodies detected in only 2% of the samples. Northern parts of country showed lowest sero prevalence while southern the highest. The observation suggested a negative impact on the survivability of *T. gondii* oocysts in drier conditions [1]. Like wise this study also shows low sero prevalence of toxoplasma among the population.

Lymphadenopathy is a common presentation in toxoplasma infection. The most commonly involved are lymph nodes of head and neck region. Cervical lymphadenopathy affects the population across all ages. In our study the age ranged from 3 patients below the age of 15 while five of them were above 50 years of age, the maximum of them were between the age group of 16- 30. Studies show that males were more affected by cervical lymphadenopathy than women. However in our study we had more female subjects.

75% percent of all lymphadenopathies are localized, with more than

50% involving head and neck area. Generally, the cause is an underlying infection, supraclavicular lymphadenopathies are however associated with malignancy. Etiology varies with different geographical areas. TB is the main benign cause in tropical areas. The common differentials for long standing cervical lymphadenopathy are tuberculosis, sarcoidoisis, toxoplasmosis, lymphoma and metastases [6].

Chronic cervical lymphadenopathy is due to either/or failure to respond to antibiotic therapy, rapid progression in size, hard, matted LNs in all cervical triangles frequently requires surgical biopsy to establish cause. To evaluate the characteristics of surgically excised cervical lymph nodes (LN) in children in a developing country, 1,332 children less than 15 years old (1,877 surgically removed cervical LNs) over a 23-year period (1976-1999) were studied. 1.5% LNs were histologically normal. 47.8% showed reactive lymphoid hyperplasia and 36.3% chronic granulomatous changes. Tuberculous lymphadenitis was confirmed in 25% cases. Other granulomatous diseases identified included Rosai-Dorfmann disease, syphilis, yaws, and toxoplasmosis. No mycobacteria other than M. tuberculosis were encountered. More than two-thirds patients with neoplastic LN involvement had a lymphoma.

They used frozen tissues of 12 lymph nodes with the histopathological triad of florid reactive follicular hyperplasia, clusters of epithelioid histiocytes, and focal sinusoidal distention by monocytoid B cells and tissues of 27 lymph nodes from patients with various other conditions to detect *T. gondii* DNA by polymerase chain reaction and serological correlation.

Twenty three immunocompromised patients were tested by IgG western blotting to identify antigens in primary *Toxoplasma gondii* infection and determine their role in diagnosis of reactivated toxoplasmosis against 23 immunocompetent persons.

Group 1 had 15 human immunodeficiency (HIV)/AIDS patients sub divided into group 1A (six patients with clinical and/or serological evidence of reactivation), group 1B (five patients with clinical evidence only), and group 1C (four asymptomatic patients).

Group 2 had eight non-HIV/AIDS immunocompromised patients with clinical and/or serological evidence of reactivation.

The concluded that detection of some low molecular weight antigens is diagnostic of reactivated toxoplasmosis and could be due to release of bradyzoites from ruptured tissue cysts. [11]

In our study, 49 of the 100 patients had granulomatous pathology, 30 were reactive and 21 had a neoplastic cause. In developing countries, because of the high prevalence of tuberculosis and lack of easy diagnostic accessibility, it is possible that many cases of toxoplasmic lymphadenitis are being diagnosed as granulomatous lymphadenitis [8].

6 cases of suspected toxoplasmic lymphadenitis were diagnosed by fine needle aspiration cytology using needle and syringe washings, air-dried smears, alcohol-fixed smears, cell blocks and confirmed by subsequent serology.

Smears showed polymorphous population of cells, germinal centers, epithelioid histiocytes with intracellular organisms resembling *Toxoplasma gondii*. Serologic testing for toxoplasma in all the cases revealed elevated titers.

Toxoplasmosis can be diagnosed by serological, bioassay, microscopy, imaging and molecular methods. The microscopy method utilizing FNAC or histology is a quick method in diagnosing toxoplasma. The method is however less reliable and not sensitive. Histopathological triad of florid reactive follicular hyperplasia, clusters of epithelioid histiocytes, and focal sinusoidal distention by monocytoid B cells is noted in most toxoplasma infected tissue. [12]

In a study conducted on 667 lymph nodes, sensitivity of the triad was found to be 62.5% (10/16) and specificity was 91.3% (21/23). The predictive value of positive tests was 83.3% (10/12) and the predictive value of negative tests was 77.7% (21/27).

None of the neoplastic diseases were positive [9]. FNAC accompanied by serology tests can clinch the diagnosis at an early stage, hence avoiding unnecessary treatments.

This triad is however also seen in infectious mononucleosis. Serological methods are the most used and considered gold standard in clinical practice. IgM levels rise in the first two weeks of infection and stays positive for the first two months.

It is considered a marker for acute infection , but it can stay prolonged in some patients making it difficult to distinguish between acute and chronic infections. There is also a possibility of false positive IgM.

However in our study none of the patients had IgM elevated though we detected elevated IgG levels suggesting past infection by toxoplasma. Of the 49 granulomatous lymphadenopathies 7 tested positive for IgG levels. (14%).

CONCLUSION:

Despite the fact that a very low prevalence was seen in our study it is recommended that a Toxoplasma be kept as a possible diagnosis when Zeilh neelsen does not detect AFB on the granulomatous lymphadenopathies.

Serologic confirmation in all suspected cases of this self-limited condition should be done, for which no treatment is necessary. Besides, cytodiagnosis can eliminate the need for hospitalization and surgery. There is also a need for larger study where both toxoplasmosis and sarcoidosis can also be taken into consideration.

1.Diagnosis on cytology

Table 1

Diagnosis	
Granulomatous	49
Reactive	30
Neoplastic	21
Total	100

2. Demography of study sample

Table 2.

Age	Male	Female
0-15	1	2
16-30	14	16
31-50	3	8
>50	2	3
Total	20	29

3. Ziehl Neelsen positivity in study population

Table 3.

Ziehl Neelsen stain	
Positive	6
Negative	43
total (n)	49

4. IgG positivity in study group

Table 4:

IgG	n
Positive	7
Negative	42
total (n)	49

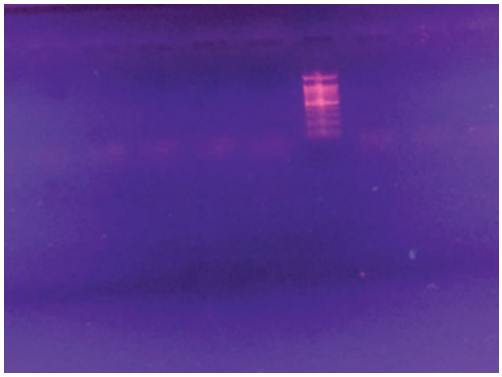

Figure: 1 Gel electrophoresis showing ladder expression of control, but absent B1 gene

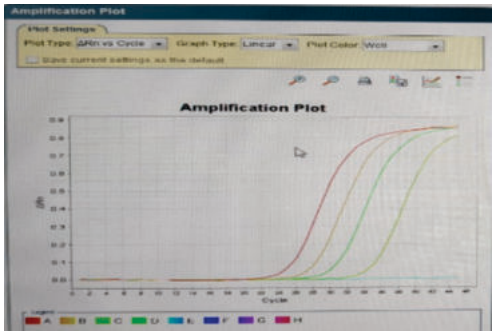

Figure: 2 Amplification plot depicting adequate technique and cycle

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