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Monoclonal Anti 38-KD Immunohistochemistry: A Novel Method for Improving the Diagnosis of Pediatric Tuberculous Lymphadenitis

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Abstract: ***Background:*** ***The clinical and histological criteria used to diagnose lymphadenitis caused by MTB organisms have low sensitivity, and poor specificity. We report a novel method for diagnosis of pediatric tuberculosis that uses Monoclonal anti 38-KD immunohistochemistry (IHC) to detect the 38- KD antigen on formalin-fixed tissue biopsies. This antigen has not been detected in non-tuberculous mycobacteria. Polymerase chain reaction (PCR) for amplification of IS6110 from DNA obtained from the biopsies was used as a gold standard in this Study.*** ***Methodology:*** ***Of 718 lymphadenopathy cases, 42 pediatric cases of granulomatous lymphadenitis with histologically suspected tuberculosis obtained from Sudan were evaluated. Z.N stain, IHC and PCR techniques were used for diagnosis. Positive and negative control for IHC and PCR were used to standardize the assays.*** ***Result:*** ***Z.N stain, IHC, and PCR positivity were observed in 1/42(2.4%), 33/42(78.6%), and 33/42(78.6%) of granulomatous lymphadenitis cases respectively. Z.N stain had sensitivity, specificity, sensitivity, specificity, PPV and NPV of 2.9%, 100%, 100%, and 55% respectively, while IHC had sensitivity, specificity, PPV and NPV of 100%, 100%, 100%, and 100%, respectively.*** ***Conclusion:*** ***IHC with anti-38-KD antiserum is a rapid, sensitive, and specific method for establishing an etiological diagnosis of Pediatric tuberculosis in histologic specimens. It can be standardized and performed by trained technicians in routine laboratory.***

Keywords: Tuberculosis, Pediatric, Immunohistochemistry, Sudan.

1. Introduction

Tuberculous lymphadenitis (TBL) is the most common clinical manifestation of extra pulmonary tuberculosis. TBL is also one of the most frequent causes of lymphadenopathy in pediatric age group [12]. Diagnosis of TBL is usually made by the relevant clinical manifestations, supported by the characteristic histopathological features of lymph node biopsies, such as presence of granulomatous lesions (with or without caseation). Granulomatous lymphadenitis has extensive differential diagnosis and is known to occur in sarcoidosis, sarcoid- like granulomatous reaction in the draining lymph nodes of malignancies of epithelial origin, fungal infection, and parasitic infection (filariasis) and lymphogranuloma venerium. At times, the histopathological features in these diseases resemble closely TBL and can pose considerable diagnostic challenge.

The diagnosis of TBL using Z.N stain is often difficult because of it has low positivity in tissue granulomas [8, 10]. Various nucleic acid amplification assays have been developed that have shown excellent sensitivity and specificity in respiratory specimens. However, use of these tests in clinical settings for non-respiratory specimens still requires validation and are still out of reach in routine diagnosis in developing countries [20]. Few immunohistochemical (IHC) studies have suggested the probable role of immunohistochemical staining in establishing mycobacterial etiology of caseating granulomas of lung, lymph nodes and tissue specimens with tuberculosis [2,7, 9, and 15] but most of these have used polyclonal antibodies resulting in false positive reactions due to antigenic cross reactivity with other bacteria and fungi. The present study was undertaken to assess the utility of immunohistochemical staining with species specific monoclonal antibody to 38 KD antigen of Mycobacterium tuberculosis complex in archival formalin-fixed, paraffin-embedded tissue sections of extra-pulmonary tuberculosis and was compared with conventional ZN staining. So far to the best of our knowledge no published study in Sudan has shown use of this antibody for the diagnosis of pediatric TBL.

2. Material and Method

A total of 718 Lymph node cases were taken for detailed study retrospectively, of which 42 cases were pediatric, histologically diagnosed as cases of TBL of various sites. Relevant clinical data regarding age, sex, site, signs and symptoms, family history, X- ray, PPD and previous history of anti-tubercular treatment of each case were retrieved from the case files of Department of Pathology, Laboratories

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administration-Khartoum state, Sudan. The histological diagnosis of lymph node tuberculosis of various sites was based on the classical caseous granulomas observed on the histopathological examination of hematoxylin and eosin stained formalin fixed paraffin embedded tissue sections. Parallel 5- micron sections were prepared from each patient's blocks and were subsequently, subjected to Z.N stain and IHC testing, also 10-micron sections were obtained and placed in Eppendrof' tube for PCR assay.

2.1 Immunohistochemistry

Immunohistochemistry was carried out using (universal IHC detection kit- NO: Ab80436) from Abeam Co.UK, which are Mouse and-Rabbit Specific HRP/DAB detection kit. The characteristic of primary antibody and the technique used for unmasking of antigenic epitopes are described in Table 1. Positive and negative control were used in this study to exclude false positive and negative result as well as to optimize the IHC assay.

2.2 PCR for IS61160

In this study rapid extraction of DNA from formalin fixed paraffin embedded tissues was carried out using ready extraction kit from (Beijing Aide biotechnology Co, Ltd) for in vitro use. Amplification of IS6110 sequence was carried out using MTB complex 390/Positive and negative control provided with the kit were used to reassess specificity of PCR product.

3. Data Analysis

Data were analyzed using a computer IBM SPSS program (version 20). The calculation of the expression of 38-KD among cases of TBL was determined by obtaining Odd ratios. Variations were determined by using Qui-square. Level of significance was set at P Value 0.05.

4. Result

In this retrospective descriptive study, (n=42) pediatric patients with lymphadenopathy were diagnosed as having TBL by histopathology depending on the presence of certain histopathological evidences (granuloma, caseous necrosis, epitheloid cells, and Langerhans giant cell). Accordingly caseous necrosis, granuloma and giant cell were evidenced in 33/42(78.6%), since epitheloid cells were evidenced in 9/42(21.4%).

In this study the age range was from 4-18 years, with age mean 11 years, the male and female ratio was equal. Most biopsies were from cervical LN (64.3%), followed by axillary LN(14.3%) and mediastinal LN, mesenteric LN, lingual LN, sub-mandibular LN, constituting 7%,4.8%, 4.8%,4.8: respectively, as indicated in **Fig.1**.

Out of (n=42) L.N cases, acid fast positivity was observed in only 1(2.4%) case of tuberculous granulomas whereas Immunoexpression of anti 38-KD was showed in 33/42 (78.6) of cases, and all positive cases showed staining deposits of brown colored product around the area of granulomas and caseation throughout the histological sections. Positive control was positive and negative control was negative.
The IS6110 PCR was positive in test of mycobacterium strains used as positive controls and the results of PCR. Out (n=42) studied lymph nodes, 33/42(78.6%) were positive for IS1160 PCR these cases were previously found as having caseous necrosis, granuloma and giant cell strong, while the remaining negative cases were previously found as having epithiloid cell only.

On the other hand when using PCR as a gold standard for comparing the other variable, accordingly the sensitivity and specificity of Z.N stain and 2.9%, 100% respectively. In contrast the sensitivity & specificity of anti 38-KD IHC was 100%, 100 respectively as shown in **Table 2**.

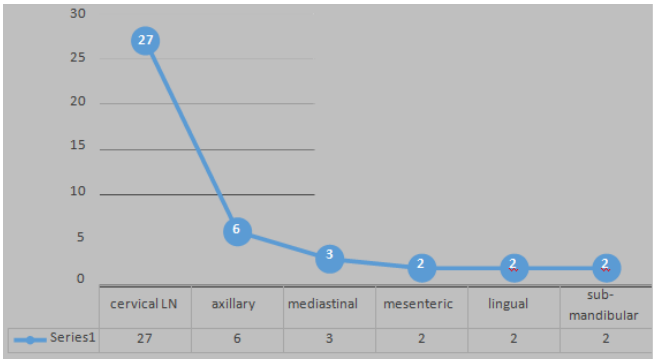


Figure 1: Description of the study population by Lymph node site

Table 1: Characteristics of the antibodies used

Antibody	Clone	Dilution	Antigen retrieval	Company	Time of reaction
Anti 38-KD	Mouse Monoclonal	1:50	HER citrate buffer pH6.0/30Min	Dako Corp Denmark	30min

***HER= heat induced retrieval**

Table 2: Diagnostic validation of Z.N stain and IHC using PCR as gold standard

Diagnostic methods	Sensitivity%	Specificity%	PPV%	NPV%

***PPV= Positive predictive value, NPV= Negative predictive value**

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Table 3: Review of Published literature on IHC studies with polyclonal and monoclonal Antibodies for Mycobacterium tuberculosis

Author &Year	*No. of cases*	*Method of IHC& Antibodies used*	*ZN positivity%*	*IHC positivity%*
Higuchi et al l981	NA*	Imrnunoperoxidase; Polyclonal anti-BCG Ab	25%	100%
Humphrey et al l987	59	Indirect Peroxidase -Antiperoxidase; Polyclonal Ab	NA	77.7%
Barbolini et al l989	23	Avidin-Biotin complex; MoAbs 60.15,61.3, & 2.16 KD	15%	100%
LuoD 1990	137 Ex*	Streptavidin Peroxidase Antiperoxidase;NA	34%	69.3%
Wiley et al 1990	34	Peroxidase -Antiperoxidase; anti-MD, BCG, MP Ab	24%	94.1
Radhakrish et al l991	10	Peroxidase - Antiperoxidase; IgG (raised in rabbit)	0%	100%
Mukherjee et al 2002	50	Avidin - Biotin Complex; Polyclonal anti- BCG Ab	44%	87%
Oliveira et al 2004	3 CR*	Immunoperoxidase (N.A)	NA	100%
Ulrichs et al 2005	NA	Imrnunoperoxidase; Polyclonal anti -BCG Ab	NA	100%
Padma-valhy et al 2005	50	Indirect Immunoperoxidase; Polyclonal anti- BCG	0%	68%
Tehmina et al 2006	55	Immunoperoxidase; Polyclonal antiMPT64	NA	60%
ManjuR et al 2007	120	Immunoperoxidase; Polyclonal anti MPT64	50%	80%
GoelMM et al 2007	69	Immunoperoxidase; Monoclonal anti38-KDAb	36%	100%
GoelMM et al 2008	113	Immunoperoxidase; Monoclonal anti38-KDAb	23%	97%
SumiS et al 2009	31	Immunoperoxidase; Polyclonal anti ESat-6	NA	87%

***NA: Data not available, CR: case report, Ex: experimental**

5. Discussion

In this study of 42 pediatric lymph node diagnosed by histopathology as having tuberculosis, according to present of certain histological evidences (granuloma, caseous necrosis, and Langerhans giant cell). Z.N stain for acid fast bacilli was positive in only in 1/42 (2.9%) cases whereas IHC showed positive Anti 38-KD staining with Mycobacterium tuberculosis antigens that included whole organisms, their fragments and debris in histological sections of 33/42 (78%) cases of extra-pulmonary tuberculosis. Low AFB positivity could be due to the fact that only the intact bacilli take up the stain or due to intensive phagocytotic activity by macrophages in tuberculous granulomas, the morphological Characteristics of AFB often get distorted, formalin fixation also may play important role in low detectability of ZN staining [18]. The positive IHC staining in areas where acid fast bacilli were absent or scarce, indicated that concentrated debris derived from mycobacteria apparently retained its antigenic property although it had lost its AFB staining property. Our study in agreement with many different published studies carried out in experimental and clinical granuloma on comparison of Z.N staining with IHC staining [1, 5, 6, 11, 13 and 14] with Z.N positivity ranging from zero percent to 44% and IHC positivity from 69% to 100%. The details of these published literatures are shown in **Table 3.**

As evident from **Table 3,** majority of the workers [4, 15, 16, and 19] have used anti-BCG, anti MTB64 and anti ESat-6 polyclonal antibodies, either raised in house or commercially available for IHC Staining. Barbolini et al 1989 [3] experimented with four types of monoclonal antibodies raised in mice against different proteins of Mycobacterium tuberculosis and observed that antibody 61.3 to 35 KD protein of Mycobacterium tuberculosis was species specific for Mycobacterium tuberculosis complex and was not reactive to Mycobacterium kansasii. In our study, species specific monoclonal antibody was directed towards a 38 KD protein of Mycobacterium tuberculosis complex showing 100% sensitivity and specificity with 100% PPV and NPV when compared with PCR which used as gold standard in this study, this finding to some extend in agreement with study carried in 2008 by Goel MM et al [17]. The classical histological picture of tuberculous granulomatous inflammation is not a diagnostic problem in a tissue biopsy. However, when the sections show non-caseous epithelioid granulomas mimicking tuberculosis, which is about 9/42(21.4%) of our biopsies, it poses a diagnostic dilemma. The positive IHC with species specific anti 38-KD in these cases will rule out the differential diagnosis of sarcoidosis or other non-specific tuberculoid granulomas.

6. Conclusion

IHC with species-specific monoclonal antibodies to 38 KDa protein of Mycobacterium tuberculosis complex may be an efficient diagnostic adjunct to conventional Z.N staining for the diagnosis of tissue granuloma of extra-pulmonary tuberculosis. The technique is simple, sensitive and specific. This will also help in clinical decision-making and in reducing the usual practice of prescribing empirical anti-tuberculous treatment based on clinical suspicion alone in the absence of demonstrable evidence of tuberculous infection.

7. Further Scope

Diagnosis of pediatric TBL is a challenge. The clinical criteria used for diagnosis have poor sensitivity and specificity and may lead to over-diagnosis, especially in countries with high endemic rates of tuberculosis. However, Further studies are highly recommended to elaborate the pitfalls in IHC for diagnosis of tuberculosis, and how to skip them to make a more rapid and accurate diagnosis for TBL.

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