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Diagnostic Utility of ZN, IH and PCR in Detection of Tuberculous Lymphadenopathy: A retrospective Study from Sudan

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Abstract: ***Background:*** ***Lymph node tuberculosis (LNTB) is the leading cause of extra pulmonary tuberculosis and is the most frequently identified type in Khartoum state, Sudan. Conventional diagnosis has serious limitations for rapid detection of LNTB in clinical samples. Therefore, the aim of this study was to assess the diagnostic utility of IH and ZN, since they are the routine used methods.*** ***Methodology:*** ***Out of 718 lymphadenopathy specimens, 161/718 (22.4%) lymph nodes tissue biopsies that were previously diagnosed by conventional histopathology as having tuberculosis were reinvestigated. The specific monoclonal anti 38KD was used to detect MTB by IHC, and IS6110 sequence for MTB was used to perform PCR. IS6110 PCR assay was performed in comparison with the histopathology diagnosis, Z.N stain and IHC.*** ***Results:*** ***ZN stain was positive in 4/161 (2.5%) of the cases, since, PCR and IHC were positive in 135/161(83.85%) and 129/161(80.12%) of the cases respectively.*** ***Conclusions:*** ***IHC and PCR have high sensitivity in detection of lymph node associated MBT. It advisable to use these techniques before reporting the negative results.***

Keywords: Tuberculosis, PCR, Immunohistochemistry, ZN, Sudan

1. Introduction

Tuberculosis (TB) remains a major global health problem. In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people) about 75% of these cases were in the African Region. [1]

Most TB cases and deaths occur among men, but it remains among the top three killers of women worldwide. There were an estimated 410 000 TB deaths among women in 2012, including 160 000 among HIV-positive women. In contrast there were an estimated 530 000 TB cases among children (under 15 years of age) and 74 000 TB deaths (among HIV-negative children) in 2012 (6% and 8% of the global totals, respectively).The majority of cases worldwide were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively. [1]

The situation in Sudan is even grimmer. In 2012 the country has Mortality (excludes HIV+TB) 8 (per 100 000 population), Mortality (HIV+TB only) 5.2 (per 100 000 population), Prevalence (includes HIV+TB) 207 (per 100 000 population) Incidence (includes HIV+TB) 114 (per 100 000 population), Incidence (HIV+TB only) 12 (per 100 000 population). [2] In Sudan approximately bout 74% of tuberculosis is pulmonary and the rest is extra pulmonary, but this data can vary from country to country. [3]

Lymph node is the leading cause of extra pulmonary tuberculosis, the most frequently extra pulmonary TB identified form is Lymph node tuberculosis (LNTB). [5] The diagnosis of LNTB, apart from clinicial evaluation, depends on several techniques like conventional Hematoxylin and Eosin (H&E) stain, the Ziehl Neelsen (ZN) stain, Immunohistochemistry (IHC) & Polymerase chain reaction (PCR). Each of these techniques has advantages and limitations. [6]

This study aimed to test the suitability of these protocols for the detection of mycobacterium tuberculosis in histological section.

2. Materials and Methods

This is descriptive retrospective study, conducted in Sudan at Khartoum state during period 2012 -2013, aimed to find out a novel technique for detection of mycobacterium tuberculosis in formalin fixed tissue. Out of (718) lymphadenopathy specimen, One hundred sixty one lymph node biopsies were retrieved, which were previously diagnosed as tuberculous lymphadenitis. All Information's including demographic characteristic like age, gender, and site of lymph node biopsy regarding each patient were obtained from each patient's file.

The specimens were fixed in 10% formalin and then processed by tissue processing machine using the adopting 24-hour scheduling. [6] Parallel, 5-micron tissue sections for H&E, Z.N stain and IHC were prepared from each patient's

block using Rotary Microtome, also 10 Micron tissue sections for PCR assay were obtained and placed in Eppendorf" tube. Carry-over tissue contamination was prevented by cleaning the blade with 96% ethanol after sectioning each sample.

Hematoxylin and Eosin was done to evaluate morphological features (granuloma formation and caseation), Z.N stain was done to look for acid fast bacilli. [6]

2.1 Immunohistochemistry:

Immunohistochemical stain was carried out by the standard Avidin Biotin Complex (ABC) method using Abcam EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436), (universal immunohistochemical detection kit). Monoclonal anti-38KD antibody (Source DAKO, Denmark) is a Mouse anti-human MTB immunohistochemistry monoclonal antibody, was used as the primary antibody. Positive control to exclude false negative result as well as to detect the reactivity of staining solution was used in this study, also negative control to exclude false positive result was used to optimize the immunohistochemical assay.

2.2 Polymerase chain reaction for IS6110 sequence:

The DNA extraction was done for each samples by kit for rapid extraction of formalin fixed paraffin embedded tissues, of (Beijing Aide Lai Biotechnology Co, Ltd,) Manufacturer Company for in vitro use.

MTB complex 390/750 IC kit from (Sacace™ biotechnologies co. Italy) with specificity 100% is an in vitro nucleic acid amplification test for qualitative detection of mycobacterium tuberculosis was used in this study. All the tubes containing extracted DNA required for the amplification subsequently it were subjected to PCR technique for detection of the IS6110 oligonucleotides of MTB using same procedure described by company. The specificity of PCR product was reassessed by using positive and negative control provided with kit.

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 LNTB was determined by obtaining Odd ratios. Variations were determined by using Qui-square. Level of significance was set at P Value 0.05.

4. Results

In this retrospective descriptive study, (n=161) patients with enlarged lymph nodes were diagnosed as having lymph node tuberculosis by histopathology. These patients were further divided into two groups according to the presences of strong and weak tuberculosis histopathological evidences. Those showing histopathological pattern containing (giant cells + granuloma + caseation) were considered as strong evidence, and the other showing less evidences were considered as weaker evidence. Accordingly, the strong evidence (positive) was used as a gold standard for comparing the other variables, as shown in **Table 1**. Of the (n=161) patients, 118 (73%) were categorized as having strong evidences (positive) and the remaining 43 (27%) were detected with weaker evidences (positive), cases.

Out of the 161 studied lymph nodes, only 4 (2.5%) lymph node were positive with Z.N (in all cases more than 5 bacilli were seen). The entire 4 Z.N positive were previously found as strong evidence positive.

Immunoexpression of Monoclonal anti 38-KD was positive in 129 (80%) of cases as shown in **Figure 1**. Out of entire 129 positive cases 100 (62%) were identified as strong evidence positive and the remaining 29 (18%) were at weak evidence level. Positive and negative control were positive and negative respectively.

The IS6110 PCR was positive in test of mycobacterium strains used as positive controls and the results of PCR are shown in **Figure 2**. Out (n=161) studied lymph nodes, 135 (84%) were positive for IS1160 PCR and remains were negative, from the entire 135 PCR positive, 106 cases were previously found as strong evidence positive and 29 was found weak evidence of which . Of remaining 26 (16%) PCR negative specimen only 12 specimens were showed strong evidence for TB, and 14 were weak evidence.

on the other hand when using PCR as a gold standard for comparing the other variable, accordingly the sensitivity and specificity of histopathology diagnosis & Z.N stain were 78.5%, 46.1% ($\chi 2 = 11.6$, $p < 0.05$) and 3.0%, 100% ($\chi 2 = 0.790$, $p > 0.05$) respectively. In contrast the sensitivity & specificity of anti 38KD IHC was 95.5%, 100% ($\chi 2 = 124.9$, $p < 0.05$) respectively as shown in **Table 2**.

Table 1: Compression between H&E MTB evidence and other techniques used

		Z.N		IHC		PCR	
		+Ve	*-Ve*	*+Ve*	*-Ve*	*+Ve*	*-Ve*
H.E	Strong MTB Evidence	4	114	100	18	106	12
	Weak MTB Evidence	0	43	29	14	29	14
Total of (*n*=161)		4	157	129	32	135	26
Total		*n*=161		*n*=161		*n*=161	

Table 2: Diagnostic validation of different tests using PCR as gold standard

Diagnostic method	*Sensitivity %*	*Specificity %*	*PPV%*	*NPV%*	*χ2*
H&E MTB evidence	78.5%	46.1%	100%	32.5%	11.6
Z.N Stain	3%	100%	100%	16.7%	0.790
IHC	95.5	100%	100%	81%	124.9

PPV = positive predictive value, NPV = negative predictive value, χ2 = chi square test of significance.

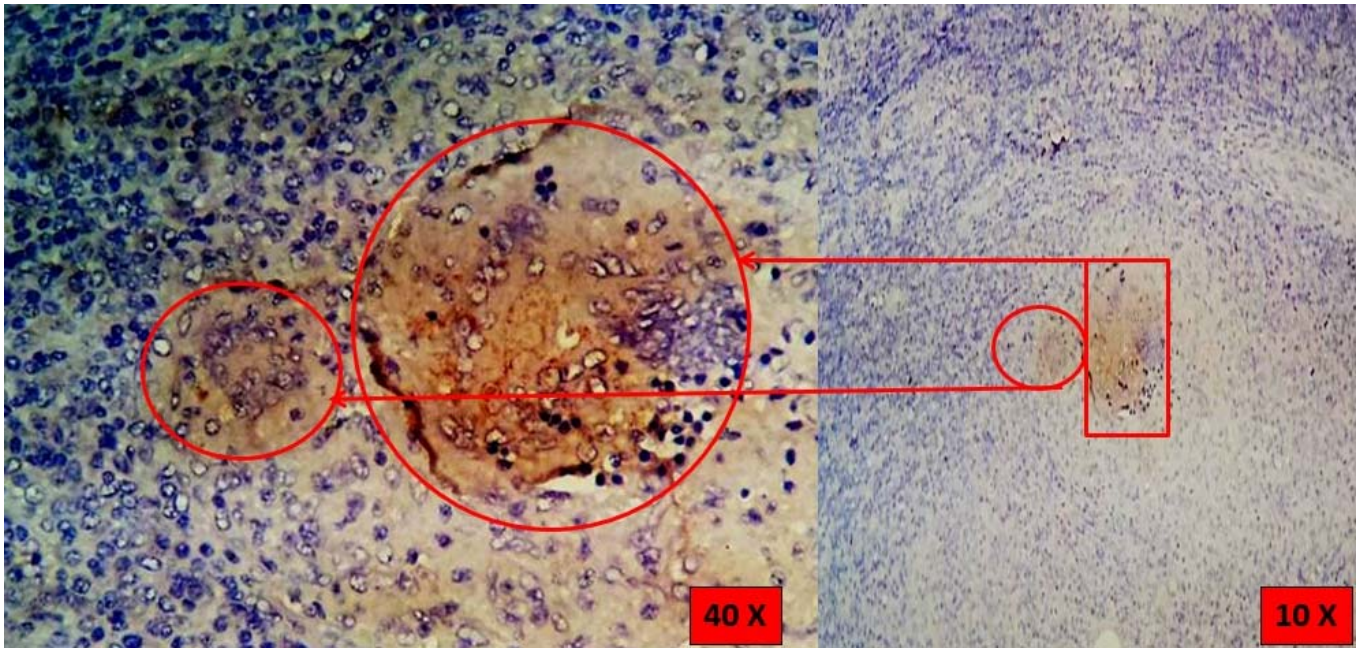

Figure 1: Strong and granular immunohistochemical stain of giant cell by anti-38 KD.

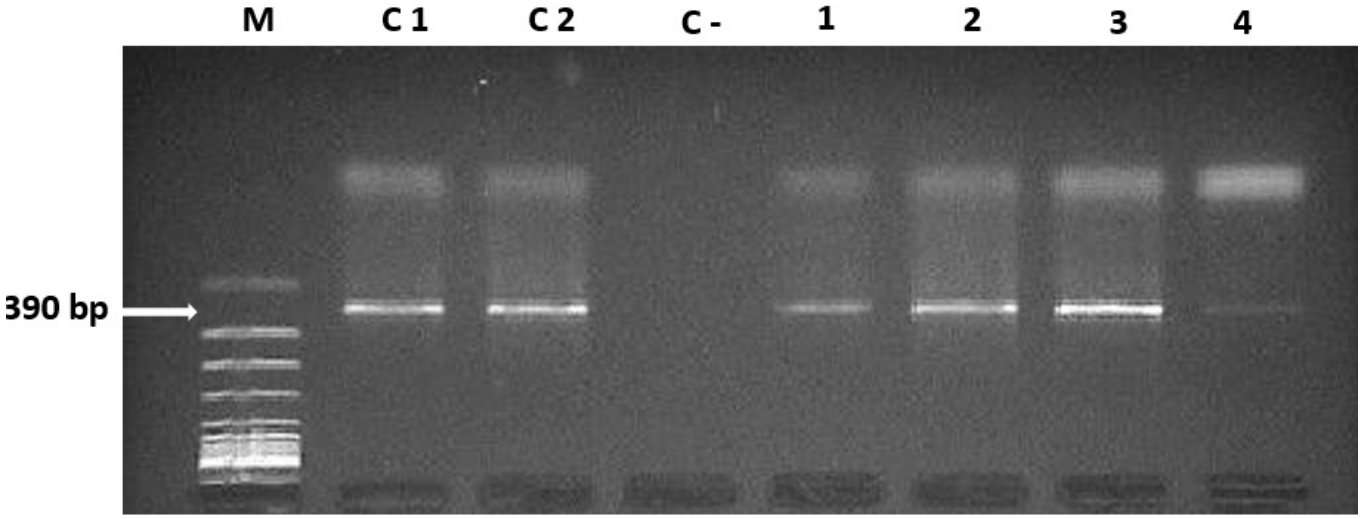

Figure 2: PCR products of the sequence IS6110 were analyzed on 2.0% agarose gel electrophoresis followed by ethidium bromide stain

M= Marker. C1, C2= Positive control. C- = DNA buffer. 1, 2, 3 and 4 Positive samples

5. Discussion

Extra pulmonary tuberculosis is frequently making challenges and there is still difficulty to differentiate it from other diseases and is often misdiagnosed. [7] Various methods for diagnosis used in different countries could be one reason for the high levels of LNTB found. Fukushima and collaborators in Japan [8] Availability of a rapid, sensitive, specific and reliable diagnosis is an essential element in the management of LNTB.

In developing countries the laboratory diagnosis of LNTB is largely based on histopathology morphological evidence (it cannot differentiate changes caused by M. tuberculosis, non-tuberculous mycobacteria or other granulomatous diseases) which is not good, followed by Z.N stain to confirm the presence of bacilli, but Z.N staining has very low sensitivity and often is less specific in histological section than molecular tests [9, 10].

Recently, many complementary diagnostic tools have increasingly been developed for the most important species of mycobacteria. [11] MTB IS6110 oligonucleotides is an attractive target for PCR amplification, and has been tested in intestinal TB and Crohn's disease. [12] The 38-KD protein is one of the most important antigens of Mycobacterium tuberculosis, it actively secreted and attached to the surface of the mycobacterial cell by a lipid tail that may also be responsible for binding of carbohydrate to the protein, this protein recently used to produce Monoclonal anti 38KD for immunohistochemical assay. [13]

In this study all study subject (n=161) were previously diagnosed as tuberculous lymphadenitis, depending on the presence of different histological evidence, these evidence

were reassessed and study subject subdivided in to two group depending on the presence of strong histological features. Out of the 161 TB patients, 118 (73%) were found with strong histological evidence of TB and remaining were weak, such findings have been previously reported by Drobniewski FA and et al. [14]

This study came out with the fact that; the acid fastness of MTB in histological section is very poor. Of (n=161) patients only 4 (2.5%) patient's were found to be TB positive with Z.N, this finding is in agreement with Hussain et al. [15] different studies carried out by Kamboj et al, have reported a wide range of Z.N positivity ranging from as low as 0% to as high as 75% [16]

As well as, the present study illustrated the importance of immunohistochemistry in identification of LNTB, the results look similar to those obtained by Tehmina Mustafa et al. [17] Of (n=161) cases 129 (80%) of lymph node showed positive expression for anti 38 KD IHC, and this to a great extend is similar to the study of Tehmina Mustafa and et al. [17] Also our result is to some extend goes with the results obtained by Manju R Purohit et al who used anti MPT64 in detection of LNTB by IHC. [18, 19]

In the present study IS6110 oligonucleotides DNA 390 bp with internal control 750 bp for PCR assay were used, following the same sequence as those used in the PCR test described by Lefmann et al. [25] and Naser et al. [26] who reported high sensitivity and specificity in detection of LNTB .

Our PCR results showed higher sensitivity and specificity in the detection of MTC in comparison to ZN staining such finding have been previously reported by Juan Rodriguez et al [21] who IS6110 oligonucleotides DNA in detection of LNTB [22,23]. Fukusima et al. [8] also reported same sensitivity and specificity for this oligonucleotide.

Here PCR was used as gold standard for comparing the other variable. Accordingly we found that the sensitivity and specificity of H&E MTB evidence were 78.5% and 46.1% respectively with negative predictive value (NPV) 32.5%, the sensitivity and specificity of Z.N stain were 3% and 100% respectively with NPV 16.7, this finding support several studies [15,19]. In contrast we found that immunohistochemistry has sensitivity 95.5% and specificity 100% with NPV 81%, similar results obtained in study elsewhere [20].

6. Conclusion

IHC with anti 38KD antiserum and PCR with IS6110 oligonucleotides are rapid, sensitive, and specific methods for establishing the diagnosis of tuberculosis in histologic specimens. Immunohistochemistry has the advantages over PCR of being robust, quicker, and cheaper, and it can be used in high-endemic countries. Therefore it is advisable to use IHC before reporting the H&E and Z.N stain negative results.

7. Future Scope

However, Lymphnode associated TB remains a challenging diagnosis for both clinicians, and pathologist. These diagnostic difficulties, the institution of appropriate therapy is often delayed in patients with TB, resulting in increased morbidity and mortality, whereas patients without TB may receive unnecessary presumptive treatment for several weeks. Therefore, the utility of nucleic acid amplification tests in the setting of TB should be extensively evaluated, to achieve a goal that enabling the clinician to make a more rapid and accurate diagnosis.

References

[1] World Health Organization (WHO), "Golbal tuberculosis report 2013. Eighteeth Global annual report'' http://www.who.int/tb/publications/global_report/en/.
[2] World Health Organization (WHO), ''Tuberculosis profile 2013'' [Online] https://extranet.who.int/sree/Reports?op=Replet&name=%2FWHO_HQ_Reports%2FG2%2FPROD%2FEXT%2FTBCountryProfile&ISO2=SD&LAN=EN&outtype=html.
[3] Tajeldin M Abdallah and Abdel Aziem A Ali, "Epidemiology of Tuberculosis in Sudan,'' *Asian Pac J Trop Biomed*, vol.12, no. 2, pp. 999-1001, 2012. [PubMed].
[4] Hargreaves JR, Boccia D, Evans CA, Adato M, Petticrew M, "The social determinants of tuberculosis; from evidence to action,'' *Am J Public Health*, vol. 101, no. 4, pp. 654-662, 2011. [PMC free article][PubMed].
[5] Kishore V, Aparna S, Prasad C, Srinivas A, Triveni B, Gokhale S, Krishna KV, "Mycobacterial culture of fine needle aspirate - A useful tool in diagnosing tuberculous lymphadenitis,'' *Indian J Med Microbiol*, vol. 26, pp. 259-261, 2008.
[6] Bancroft JD, Stevens A. Theory and Practice of Histological Techniques, 6th ed, Edinburgh, Churchill Livingstone Publications, 2007.
[7] Bayazıt Y, Bayazıt N, Namiduru M, "Mycobacterial Cervical Lymphadenitis,'' *ORL J Otorhinolaryngol Relat*, vol. 66, pp. 275-280, 2004.
[8] Fukusima Y, Shiobara K, Shiobara T, Tetawaki M, Arizal M, Fukushima F, Yamada I, Hirata H, Sugiyama K, Fukuda T, "Patients in whom active tuberculosis was diagnosed after admission to a Japanese university hospital from 2005 through 2007,'' *J Infect Chemother*, vol. 17, pp. 652-657, 2011.
[9] Dimoliatis ID and Liaskos CA, "Six Mantoux tuberculin skin tests with 1, 2, 5, 10, 20, and 50 units in a healthy male without side effects: Is skin reaction a linear function of tuberculin dose?, '' *Cases Journal*, vol.1, pp. 115, 2008.
[10] Portillo-Gómez L, Murillo-Neri MV, Gaitan-Mesa J, Sosa-Iglesias EG, "Nested polymerase chain reaction in the diagnosis of cervical tuberculous lymphadenitis in Mexican children,'' *Int J Tuberc Lung Dis*, vol. 12, pp. 1313-1319, 2008.

[11] Park DY, Kim JY, Choi KU, Lee JS, Lee CH, "Comparison of polymerase chain reaction with histopathological features for diagnosis of tuberculosis in formalin-fixed, paraffin-embedded histologic specimens, " *Arch Pathol Lab Med*, vol. 127, pp. 326-330, 2003.
[12] Jin XJ, Kim JM, Kim HK, Kim L, "Histopathology and TB-PCR kit analysis in differentiating the diagnosis of intestinal tuberculosis and Crohn's disease,'' *World J Gastroenterol*, vol .16, pp. 2496-2503, 2010.
[13] Morten H and Harald G. W, "The 38-kDa Protein of Mycobacterium tuberculosis," *The Journal of Infectious Diseases*, Vol. 166, no. 4, pp. 874-884, 1992.
[14] Drobniewski FA, More PG, Harris GS, "Differentiation of Mycobacterium tuberculosis complex and nontuberculous mycobacterial liquid cultures by using peptide nucleic acid-fluorescence in situ hybridization probes,'' *J Clin Microbiol*, vol. 38, pp. 44-447, 2000.
[15] Hussain GA, Akram SN, Ibrahim G, "Screening for Tuberculosis and Its Histological Pattern in Patients with Enlarged Lymph Node,'' *Pathology Research International*, Volume 2011, doi:10.4061/2011/417635, Article ID 417635.
[16] 3S. Kamboj, M. M. Goel, P. Tandon, S. M. Natu, and P. Nath, "Correlative study of histopathology and bacteriology in patients of tubercular lymphadenitis," *The Indian Journal of Chest Diseases & Allied Sciences*, vol. 36, no. 4, pp. 187–191, 1994.
[17] Tehmina Mustafa, Harald G Wiker, Sayoki GM Mfinanga, Odd Mørkve and Lisbet Sviland, "Immunohistochemistry using a Mycobacterium tuberculosis complex specific antibody for improved diagnosis of tuberculous lymphadenitis,'' *Modern Pathology*, vol. 19, pp. 1606–1614, 2006.
[18] Manju R Purohit,Tehmina Mustafa, Harald G Wiker, Odd Mørkv, and Lisbet Sviland, "Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64,'' *Diagnostic Pathology*, vol. 36, no. 2, pp. 1746-1596, 2007.
[19] Gutierrez CMM Garcia M, J,F, "Comparison of Ziehl-Neelsen staining and immunohistochemistry for detection of Mycobacterium bovis in bovine and caprine tuberculosis lesions,'' *J Comp Pathol*, vol. 109, pp. 361-370, 1993.
[20] Haines DM, Chelack BJ, "Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology,'' *J Vet Diagn Invest*, vol. 3, no. 1, pp. 101-112, 1991.
[21] Juan Rodriguez-Nuñez, Francisco J. Avelar, Francisco Marquez, Bruno Rivas-Santiago, Cesar Quiñones, Alma L. Guerrero-Barrera, "Mycobacterium tuberculosis complex detected by modified fluorescent in situ hybridization in lymph nodes of clinical samples,'' *J Infect Dev Ctries*, vol. 6, no. 1, pp. 58-66, 2012.
[22] Quan C, Lu CZ, Qiao J, Xiao BG, Li X, "Comparative evaluation of early diagnosis of tuberculous meningitis by different assays," *J Clin Microbiol*, vol. 44, pp. 3160-3166, 2006.
[23] Viera AJ, Garrett JM, "Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology," *J Clin Microbiol*, vol. 43, pp. 4357-4362, 2005.
[24] Park DY, Kim JY, Choi KU, Lee JS, Lee CH, Sol MY, Lee JS, Suh KS, "Comparison of polymerase chain reaction with histopathological features for diagnosis of tuberculosis in formalin-fixed, paraffin-embedded histologic specimens," *Arch Pathol Lab Med*, vol. 127, pp. 326-330, 2003.
[25] Lefmann M, Schweickert B, Buchholz P, Gobel UB, Ulrichs T, Seiler P, Theegarten D, Moter A, "Evaluation of peptide nucleic acid-fluorescence in situ hybridization for identification of clinically relevant mycobacteria in clinical specimens and tissue sections," *J Clin Microbiol*, vol. 44, pp. 3760-3767, 2006.
[26] Naser SA, Shafran I, Schwartz D, El-Zaatari F, Biggerstaff J, "In situ identification of mycobacteria in Crohn's disease patient tissue using confocal scanning laser microscopy," *Mol Cell Probes*, vol. 16, pp. 41-48, 2002.

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