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**Research Article** 

# Application of Bleach Concentration Method in Tissue Samples Received for Diagnosis of Extra Pulmonary Tuberculosis Diagnosis

#### Pranali Pingle\*

Corresponding author: Pranali Pingle, Microbiologist, Medicare Hospital, Indore, India, Tel: 9827096447; E-mail: pranalispingle@yahoo.com

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#### Abstract

Extra pulmonary Tuberculosis (TB) comprises 15% of the total tuberculosis cases. In cases of suspected extrapulmonary tuberculosis, rapid and accurate laboratory diagnosis is of prime importance, since traditional techniques of detecting acid-fast bacilli have limitations. The major difficulty with mycobacteria in tissue samples is achieving optimal cell lysis. A comparison of two methods, pretreatment of tissue with 4% Sodium Hypochlorite in Bleach concentration method and pretreatment with petroff's method before culture on Lowenstein Jensen medium, was conducted on 18 extrapulmonary tissue specimens collected from different sites of suspected TB patients to evaluate the use of Bleach concentration method in tissue samples. The aim of this study is to apply this method for demonstration of AFB in tissue samples obtained from extrapulmonary sites and to correlate with Ziehl Neelson staining and LJ culture. A total of 18 tissue samples were studied from clinically suspected cases of Extra pulmonary TB which included endometrial tissue (15), (1) from kidney and (1) from brain. All the samples were processed for conventional ZN staining, bleach concentration method, PCR and AFB culture on LJ media. Out of 18 samples none were suggestive for TB by ZN staining, while 1(5.55%) was positive by PCR, 3(16.66%) were suggestive by bleach concentration method. However to the best of our knowledge this is the pioneer study applied to the tissue samples and the results of the present study shows improved detection of AFB.

**Keywords:** Extrapulmonary tuberculosis; Bleach concentration method; LJ culture; PCR

## Introduction

Tuberculosis, a leading cause of death, infects more than a third of the world's population [1]. India alone accounted for 2.0-2.5 million cases in 2010, thus contributing approximately 26% of all TB cases worldwide [2]. EPTB has become more common since the advent of human immunodeficiency virus (HIV) infection [3]. EPTB constitutes about 15-20% of TB cases and can constitute up to 50% of TB cases in HIV-infected individuals [4]. Conventional methods for the diagnosis of tuberculosis include smear and culture for Mycobacterium tuberculosis. Ziehl-Neelson staining for acid-fast bacilli requires 104-106 bacilli/ml of tissue or fluid specimens to give a positive result [5,6] Although culture for mycobacterium is more sensitive, it still needs 101-102 bacilli/ml of sample for the diagnostic yield and requires two to four weeks for the growth of M. tuberculosis. Diagnosis of tuberculosis from tissue samples is usually made by histopathological examination (HPE) that depends on the presence of granulomatous inflammation and caseous necrosis. It needs high expertise and the final reporting takes more than one week. A diagnostic method that is less time-consuming and at the same time has high sensitivity and specificity is therefore desirable [7]. Advanced molecular methods such as Polymerase Chain Reaction (PCR), a type of nucleic acid amplification system, have shown very promising results for early and rapid diagnosis of the disease due to its detection limit of one to ten bacilli in various clinical samples. IS6110 has been proved to be a good target because of the presence of multiple copies of this insertion sequence (1-20) in most strains of M. tuberculosis complex [8,9]. However, few studies from different geographical

regions of the world have reported that some clinical isolates have either a single copy or no copy of IS6110 which leads to false negative results [10-12]. Newer molecular techniques, such as polymerase chain reaction (PCR), although rapid; are too costly to be routinely used in the settings where most TB cases occur [10]. The usefulness, priority and scope of various techniques used in TB bacteriology depend on the epidemiological situation prevailing in individual countries and on the resources available. In most low income countries, the only practically available bacteriological method for diagnosing extrapulmonary tuberculosis is direct smear microscopy for AFB of the sample from the lesion. Therefore, there is a need to detect methods for improvement of diagnosis of tuberculosis by techniques that are appropriate in developing countries. Various studies have shown bleach concentration method is cost effective, sensitive, versatile and safe procedure for demonstration of tubercle bacilli and is very valuable in diagnosing case of extra pulmonary tuberculosis [13-16] and would benefit the patients to receive an early and specific treatment. Based on the above literature we have used the bleach concentration method in tissue samples which is in its type the first study. Using tissue samples as a source, the bleach concentration prior to Zeihl-Nelson (ZN) staining was evaluated to see the increase in positivity of AFB as compared to direct ZN staining. Further the results were also compared by culture on Lowenstein-Jensen (LJ) media and PCR.

## **Materials and Methods**

This study was done in the period from August 2012 – December 2013 in Medicare hospital.

Microbiologist, Medicare Hospital, Indore, India

There were 18 samples processed during this period, the samples included endometrium tissue, Kidney tissue and brain tissue suspected for TB. Samples were collected by the doctors in operation theatre in a sterile container having sterile distilled water, and precautions were taken to avoid skin contamination, the samples were kept at 4°C before processing. All the tissue biopsies were homogenized in sterile pestle and mortar followed by centrifugation at 6,000 rpm for 10 minutes. The sediment was collected in sterile container, and is then divided into three parts.

The first portion was used for direct ZN staining and bleach concentration followed by ZN staining. ZN staining -ZN staining was performed by conventional method.

Bleach concentration method- It was performed using slight modification of the original method. Sterile, disposable test tubes were used for bleaching. In this approximately 1 ml of the sediment was mixed with 1 ml of commercially available 4% sodium hypochlorite (merck). After thorough mixing the mixture was incubated for 30 minutes at room temperature with frequent mixing at intervals. An equal volume of commercially available distill water was then added and mixed thoroughly using vortex mixer or disposable sterile plastic pipette and then centrifuged at 3000 g for 15 minutes. The supernatant was discarded, and smears were prepared using one drop of the sediment, air dried, heat fixed and stained by ZN staining technique. As a control 2 ml of distilled water was centrifuged and the sediment was stained by ZN staining to rule out any error due to contamination while testing each specimen. After conventional ZN staining and bleach concentration methods the smears were examined under 100 oil fields for the presence of AFB which is the standard procedure LJ culture- The second portion was used for culture in which the deposits were decontaminated by adding an equal volume of molar sodium hydroxide and mixed for 30 minutes. After centrifugation the sample were neutralized by 8% HCl with the help of neutral red and then cultured on LJ slant. PCR- The third portion was used for DNA extraction by Qiagen kit and followed by multiplex PCR for the IS6110 gene specific for M. tuberculosis.

DNA extraction- Tissue which was obtained in sterile saline were only accepted, using gloves and a sharp scalpel blade against the wall of the container the tissue were finely minced. The minced tissue was suspended in 1 ml of sterile distill water in a 1.5 ml vial, and centrifuged at 1000 rpm for 5 min so that large particles get sedimented. 500  $\mu$ l of the supernatant was taken and centrifuged at 6000 g for 10 min, the supernatant was decanted and pellet was treated with NALC-NaOH method for the decontamination and liquefaction to obtain the pellet for DNA extraction. (11) The bacterial pellet was resuspended in 180 ul buffer ATL (supplied in QIAamp Tissue Kit, QIAGEN GmbH, Hilden, Germany). The extraction of TB DNA was performed according to QIAGEN kit protocol. The eluted DNA can be stored at -20°C until use in PCR.

PCR assay. The SeeplexR MTB ACE detection uses multitarget (IS6110 and MPB64) and carries out both IS6110 and MPB64 PCR. The PCR mastermix was prepared according to standard protocol of the kit which contained primer pairs, DNA polymerase, buffer containing dNTPs, MgCl<sub>2</sub> and stablizersand 8-MOP solution (which prevents carry over contamination). This 15  $\mu$ l of mastermix is mixed with 5  $\mu$ l of sample's nucleic acid. The PCR conditions for DNA amplification were an initial denaturation step of 94C for 15 min, followed by 40 cycles of 94C for 0.5 min, 62C for 1.5 min, 72C for 1.5 min. and a final extension step at 72C for 10 min. The PCR procedure was accomplished with a thermocycler TC 9600 (Perkin-Elmer Cetus).

After detection step, irradiate UV light (365 nm) onto PCR product for 20 min to prevent carry over contamination. Each experiment included positive and negative control tubes. The products of amplification were then analyzed by agarose gel electrophoresis.

## Results

A total of 18 tissue samples were studied. The cases included belonged to the age groups from 24 to 45. There were 2 males (11.11%) and 16 females (88.89%). The details of different types of tissue sample are indicated in the Table 1, and about their findings are indicated in Table 2.

| Method | Samples (tissue) | No. | Percentage |
|--------|------------------|-----|------------|
| Male   | kidney           | 2   | 11.11      |
|        | brain            |     |            |
| Female | Endometrium      | 16  | 88.89      |
| Total  |                  | 18  | 100.00     |

Table 1: Genderwise distribution of Patients (tissue Sample, N=18)

| Method        | Positive | Percentage of positivity | Negative |
|---------------|----------|--------------------------|----------|
| Z-N           | 0        | 0%                       | 18       |
| TB-PCR        | 1        | 5.55%                    | 17       |
| AFB culture   | 3        | 16.66%                   | 15       |
| Bleach method | 3        | 16.66%                   | 15       |

Table 2: Detection by direct methods (tissue Sample, N=18)

Out of the sample processed, 15 were endometrial tissue 1 was tissue from kidney and 1 was from brain. The smears by conventional ZN staining were negative for all the samples (0%).

Now when the samples were processed for bleach and then stained by ZN it detected AFB in 3 samples (16.66%). Among the 3 bleach positive samples all were positive on AFB culture, 1 was positive by PCR but all were negative by conventional ZN staining.

Except the 3 samples which were positive by bleach method, there were no other samples (n=3) (16.66%) which were detected by PCR or AFB culture which is considered to be a gold standard in TB diagnosis.

## Discussion

This study was undertaken to assess the utility of various diagnostic modalities for diagnosis of Extrapulmonary tuberculosis. Also an attempt was made to compare the sensitivity of bleach concentration method with the conventional ZN staining, and culture. In this study tissue samples from different sites are taken as samples and they are tested by Zn staining, Bleach concentration method, and LJ culture.

In the present study all the tissue samples were negative by conventional ZN staining which is less as compare to Bhanu et al. [17] and Thangappah et al. [12] which shows 1.3% and 8.3% on endometrial samples by ZN staining. Whereas the study done by Ghenaat et al. [18] showed 27.5% positivity by ZN stain in lung tissue samples. Reddy et al. [19] showed 34% positivity on paraffin embedded tissue suspected for Extra pulmonary TB.

In the present study, bleach concentration method was positive in 16.66%, there was no study found to compare the bleach concentration method on tissue samples.

With the use of LJ culture, we could diagnose tuberculosis in 16.66% (n=3) wheras when compared it was more than the study by Bhanu et al. [17] (3.2%), Thangappah et al. [12] (5.6%) and Mani et al. [20] (13.6%) done on endometrial biopsy. In the study by singh et al. [21] there were 22.7% of lymphnode aspirates positive on LJ culture.

When PCR was performed on the tissue samples, 5.5% (n=1) was positive wheras when compared to Goel et al. [22] (22.2%), Khanna et al. [23] (26%), Thangappah et al. [12] (36.7%), Kohli et al. [24] (13%) the positive results were lower in this study.

The limitation of this study was that histopathology results were not compared.

#### Conclusion

To conclude, bleach method as evidenced by this study forms a cost effective, sensitive, versatile, and safe procedure for demonstration of AFB in tissue samples. On basis of the increased positivity observed by the bleach concentration prior to ZN staining it can be recommended as an initial step in the diagnosis of TB rather than conventional ZN staining. Until date, none of the available test can pick up all the cases of TB, but use of bleach concentration method with HPE, PCR and LJ will definitely increase the possibility.

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