Amer J Med Nat Sci, Volume 1; Issue 1: 1-5. ISSN: 2582-6182

Research Article

American Journal of Medical and Natural Sciences

www.ajmns.com

Evaluation of Triton Ziehl Neelsen Technique in the Detection of Intracellular *Tuberculosis bacilli* in Sputum

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Received: 12/02/2020; Revised: 23/02/2020; Accepted: 25/02/2020.

ABSTRACT

Triton X 100 reagent is a cell membrane permeabilizing agent. Its addition to conventional Ziehl Neelsen staining has been shown to improve detection of *Tuberculosis bacilli* in CSF by staining bacilli inside the inflammatory cells. We evaluated its performance in sputum in the diagnosis of pulmonary tuberculosis. We compared the Triton ZN technique with the conventional ZN method on the sputum of 68 patients suspected with pulmonary tuberculosis by overall agreement analysis. The Triton ZN technique was not superior to the conventional AFB staining method at all the tested concentrations of Triton X100 by an overall percent agreement ranging from 95.4% to 72.1%, positive percent agreement 91.65% to 9.1% and negative percent agreement 96.2% to 87.5%. Our study suggests that Triton X 100 added to conventional ZN staining does not improve detection of *M. tuberculosis* in sputum.

Keywords: Ziehl Neelsen stain; Modified AFB stain; Tuberculosis; *Mycobacterium tuberculosis;* Triton.

1. INTRODUCTION

Tuberculosis is a chronic debilitating disease which still afflicts 2.2 million people in India every year. The burden of TB remains high and many patients remain undetected until very late. These cases largely lie in the remote areas that are only provided with PHC or Sub centre with minimum capabilities. Under Revised National Tuberculosis Control Program [RNTCP] Chest X Ray and sputum staining are the principal methods to diagnose TB. These facilities are provided even in the peripheral areas. Hence low cost modifications that can improve TB detection can easily be included to the existing system with minimal investment and

high acceptability and most importantly remain accessible to the people in remote areas, who need them the most.

Ziehl Neelsen [ZN] staining of Acid Fast Bacilli is the standard staining technique to detect Mycobacterium tuberculosis bacilli [MTB] under the RNTCP in India (TB India, 2016). In 2012, Chen *et al.,* showed that the addition of Triton to ZN stain can dramatically improve the bacterial detection in the cerebrospinal fluid by staining intracellular MTB in tuberculous meningitis (Chen *et al.,* 2012). They attributed the improved detection rates to the use of TritonX100, a membrane permeabilizing agent (Koley and Bard, 2010) which was hypothesized to have allowed the carbol fuchsin dye to enter the macrophage/neutrophil cells and stain the intracellular MTB.

The new technique has not been tried on sputum to detect pulmonary tuberculosis. Improved detection of intra cellular TB bacilli by combining fluorescent acid-fast AR for staining bacteria, hematoxylin QS for staining tissue and DAPI for staining nuclei was described in a recent study (Hoff *et al.,* 2011). But the complexity of the technique with the expensive equipment and consumables required does not make this

method easily available to the resource poor remote health centers.

The objective of our study was to test this method (based on Cheng *et al.,* 2012) for detection of intracellular MTB in the sputum in patients with suspected pulmonary tuberculosis. We compared the standard ZN technique with the new technique to detect intracellular MTB in pulmonary tuberculosis. We added different concentrations of TritonX100 to the standard ZN technique guided by the work by Chen *et al.,* 2012 and Feng *et al.,* 2014 to study its role in improving the detection of intracellular MTB in sputum.

2. METHODS

At Guntur Medical College, Guntur, India from Dec 2016 to Jan 2017 we collected 68 sputum samples of patients with cough ≥ two weeks i.e. suspected pulmonary tuberculosis. Each sample was immediately processed by standard ZN technique stain (Kumar, 2012) for AFB and the new techniques which used Triton as cell permeabilizing agent. The techniques in brief-

Standard ZN technique

Preparation of bacterial smear [2X3cm] on a clean and grease free slide, using sterile technique. Air drying [15-30min] and then heat fixation. After fixation 1% Carbol fuchsin staining and heating for 8 min and then washing with clean water. 3% v/v hydrochloric acid alcohol added for decolourisation upto 5 minutes and then washing with clean water. Counter staining with methylene blue for 1–2 minute. Washing with clean water and air drying.

New Technique 1 – 0.3%TZN [Triton ZN]

Preparation of bacterial smear [2X3cm] on a clean and grease free slide, using sterile technique. Air drying [15-30 min] and then heat fixation. Addition of 0.3% Triton [in methanol] and wait for 30 min. Washing with clean water. 0.3% Triton in 1% Carbol fuchsin staining and heating for 8 min. Washing with clean water. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.

New Technique 2 – 1%TZN

Preparation of bacterial smear [2X3cm] on a clean and grease free slide, using sterile technique. Air drying [15-30 min] and then heat fixation. Addition of 1% Triton [in methanol] and wait for 30 min. Washing with clean water. 0.3% Triton in 1% Carbol fuchsin staining and heating for 8 min. Washing with clean water. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.

New Technique 3 – 10%TZN

Preparation of bacterial smear [2X3cm] on a clean and grease free slide, using sterile technique. Air drying [15-30min] and then heat fixation. Addition of 10% Triton [in methanol] and wait for 30 min. Washing with clean water. 0.3% Triton in 1% Carbol fuchsin staining and heating for 8 min. Washing with clean water. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.

New Technique 4 – 25%TZN

Preparation of bacterial smear [2X3cm] on a clean and grease free slide, using sterile technique. Air drying [15-30 min] and then heat fixation. Addition of 25% Triton [in methanol] and wait for 30 min. Washing with clean water. 0.3% Triton in 1% Carbol fuchsin staining and heating for 8 min. Washing with clean water. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.

The presence or absence of intracellular AFB was noted by examining 300 fields microscopically, using the 100 X oil immersion objective.

Statistics

We compared the new technique with conventional ZN technique by Overall agreement. [The proportion of overall agreement is the proportion of cases for which two diagnostic methods agree. With binary ratings, there are two such indices, positive agreement (PA) and negative agreement (NA). PA, for example, estimates the conditional probability, given that one of the raters, randomly selected, makes a positive rating, the other rater will also do so]. It is standard practice to compare a new diagnostic method with a Gold standard, but there is no accepted gold standard for AFB staining, hence we used the overall agreement method. It might be suggested that PCR may be considered as the standard, but PCR detects the DNA i.e. it can be a Gold standard for the diagnosis of TB. It however is not a staining technique and the detected particle is different in PCR, the TB DNA. In the staining technique we detect the bacteria but not it's DNA, for which there is no Gold standard. The study was approved by the Institutional Ethical Committee at Guntur Medical College, Guntur. IEC/GMC/31.

3. RESULTS

The typical smears by the five techniques are shown in the Figure 1.

The Triton ZN techniques showed comparatively lower agreement with the conventional ZN technique. Conventional ZN technique detected intra cellular bacilli in more number of cases than all the Triton modified techniques. With the increase in the concentration of Triton used, the detection grew lower (Table 1 and Figure 2).

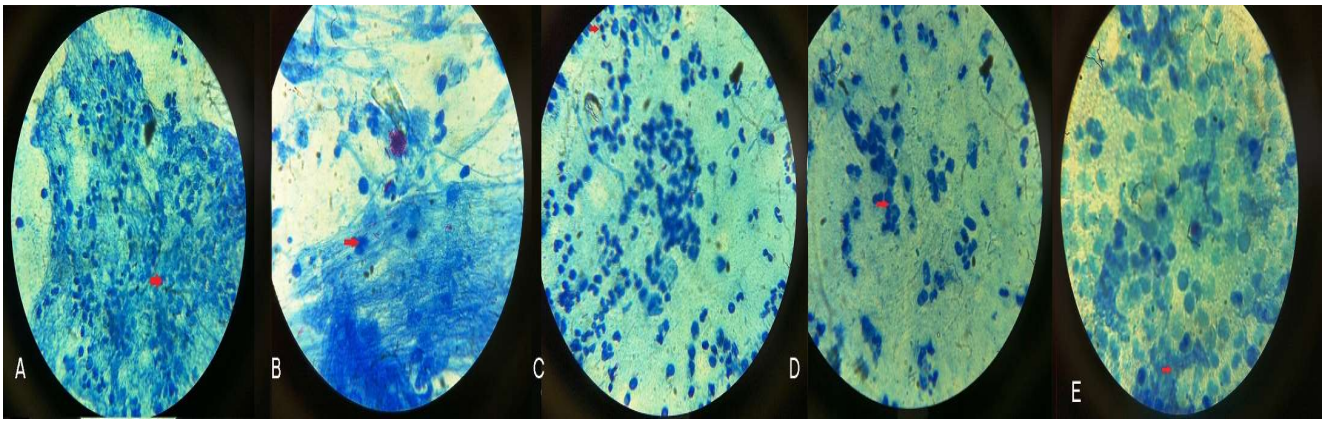

Figure 1: Typical smears by the different concentrations of Triton.

Table 1: Agreement of the Triton technique at different concentrations with the standard ZN technique.

Methods compared	Overall agreement	Positive% agreement	Negative% agreement
ZN and 0.3%TZN	95.4	91.6	96.2
ZN and 1%TZN	86.3	63.6	90.2
ZN and 10%TZN	72.1	27.3	87.5
ZN and 25%TZN	72.1	9.1	93.8

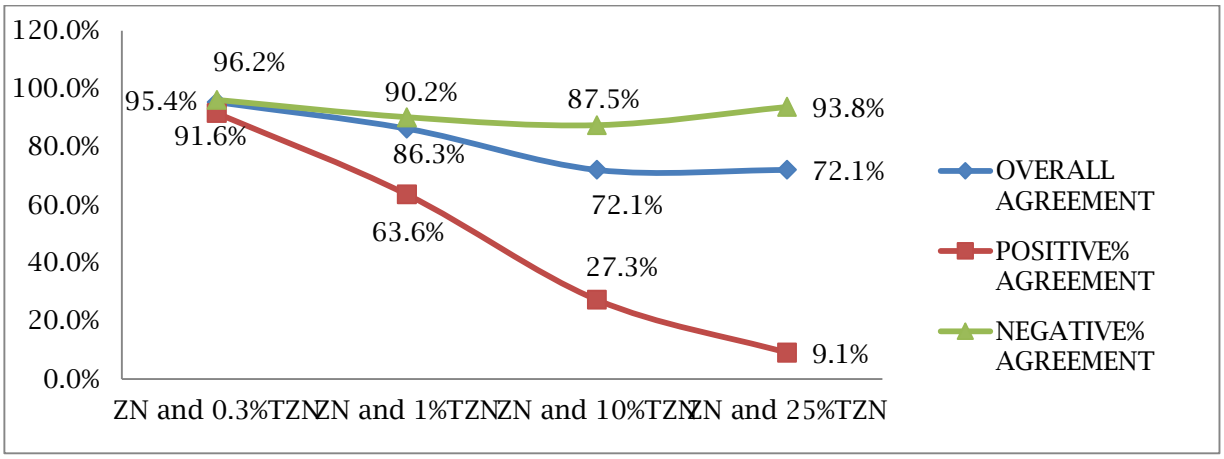

Figure 2: Decreasing trend of agreement with increase in concentration of Triton

4. DISCUSSION

We evaluated the performance of Triton modification of ZN staining technique to detect Intra cellular MTB in the sputum of patients with suspected pulmonary tuberculosis. The new technique was not superior to the conventional ZN technique.

This failure of the Triton addition technique in sputum might be due to the difference in the composition of sputum and CSF. Sputum contains variable amounts of carbohydrate, protein, lipids, DNA, α-antitrysin, LDH, lysozyme, lactoferrin and other substances. These may interfere with the actions of Triton.

In a multi centre study, Feng *et al.,* compared conventional ZN technique with Triton technique in 280 meningitis patients. In this work, the addition of triton was also accompanied by cyto-centrifugation. Cyto-centrifugation improves the sensitivity of dyeing methods by creating a concentrated monolayer of cells by centrifugation. As our work did not include cyto-centrifugation, the results may have been affected.

Chen *et al.,* showed a high bacterial detection rate in CSF with the addition of Triton to ZN staining method to detect AFB. They claim that this might be due to staining of intracellular bacilli in the Triton technique. But in our study we could stain intracellular bacilli even by the conventional ZN stain.

Experimenting with HeLa cells, Koley *et al.,* showed that 0.17 to 0.18 mM concentration of TritonX100 for 20 min was ideal to permeabilise the cell membrane. Our work based on *Chen et al.,* did not show the advantage of triton addition, but a concentration of 0.17 to 0.18mM is ten times lower than 0.3% Triton. If the higher 0.3% concentration could not improve the detection of intracellular bacilli, the effect of much lower concentrations may not prove fruitful.

By overall agreement analysis our study shows that addition Triton dye to ZN staining technique may not improve the detection of intracellular MTB in sputum. The small sample size is a limitation of our study.

Acknowledgements

The authors are thankful to Dr. Ravi Kiran for his help with microscopy.

Declaration of Conflicting Interests

None Declared.

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Cite this article: Sridhar A and Sundarachary N. Evaluation of Triton Ziehl Neelsen Technique in the Detection of Intracellular *Tuberculosis bacilli* in Sputum. *Amer J Med Nat Sci.* 2020; 1(1): 1-5.