International Journal of Advanced Research in Medicine

E-ISSN: 2706-9575
P-ISSN: 2706-9567
IJARM 2020; 2(2): 50-56
Received: 02-12-2020
Accepted: 25-12-2020

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Triton Ziehl-Neelsen technique does not improve detection of TB bacilli in sputum

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DOI: https://doi.org/10.22271/27069567.2020.v2.i2a.276

Abstract
Background: Addition of Triton X 100 reagent to conventional Ziehl-Neelsen staining has been shown to improve detection of tuberculosis bacilli in CSF to diagnose TB meningitis. We evaluated its performance in sputum in the diagnosis of pulmonary tuberculosis.
Methods: We compared the Triton ZN technique with the conventional ZN method on the sputum of 68 patients suspected with pulmonary tuberculosis by Bland Altman agreement analysis.
Results: The Triton ZN technique detected lower number of bacilli than the conventional AFB staining method at all the tested concentrations of Triton X100 by a mean difference in the AFB score ranging from 0.2 to 0.5.
Conclusions: 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, AFB stain, Tuberculosis, Mycobacterium tuberculosis, Triton

Introduction
Tuberculosis is a chronic debilitating disease which still afflicts 2.2 million people in India every year. This is a stark fact considering India was the first country to launch a National Tuberculosis Control Program in 1962 and provides free diagnosis, treatment and house to house surveillance for tuberculosis. Under Revised National Tuberculosis Control Program [RNTCP] tuberculosis detection methods have reached upto the molecular level with CB-NAAT centres established in every state of the country [1]. But 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. Chest X Ray and sputum staining are the often the only methods available to diagnose TB in the peripheral areas. In a resource poor country like India, low cost modifications to existing methods can improve the detection of TB. Such techniques 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 in all hospitals [1]. In 2012 Chen, *et al.* [2] showed that a modified ZN stain of the cerebro spinal fluid can dramatically improve the bacterial detection [by staining intracellular MTB also] in tuberculous meningitis. They attributed the improved detection rates to the use of three techniques in the new method. The first was the use of TritonX100, a membrane permeabilizing agent [3] which was hypothesized to have allowed the carbolfuchsin dye to enter the macrophage/neutrophil cells and stain the intracellular MTB. They used a cytocentrifuge [4] [centrifuge with inbuilt slide holders] to capture all the inflammatory cells, which are notoriously low in CSF even in meningitis. And they also used poly-L Lysine coated slides which are supposed to improve the adhesion of cells to the slide, evidence for which is not clear [5, 6].
The technique has not been tried on sputum to detect pulmonary tuberculosis. If found superior to conventional ZN technique, this new method could potentially be very easily added to the existing AFB staining facilities in the remote PHC's of India with minimal investment and training. As sputum contains very high number of inflammatory cells, the advantage of concentrating the very few cells in CSF by cytocentrifuge processing may not be replicated with sputum. Poly L lysine coated slides are not superior to conventional slides

in pulmonary samples as per recent studies [6]. Hence we did not use the cytocentrifuge and poly l lysine slides which are also expensive.

The objective of our study was to test this method [based on Cheng, *et al.* for detection of MTB in the sputum in patients with suspected pulmonary tuberculosis. We compared the standard ZN technique with the new technique to detect pulmonary tuberculosis. We added different concentrations of TritonX100 to the standard ZN technique guided by the work by Chen and Feng [7] to study its role in improving the detection of MTB in sputum.

Methods

From Dec 2019 to Jan 2020 at Guntur Medical College, Guntur, India 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 [8] for AFB and the new techniques which used Triton as cell permeabilizing agent. Triton X-100 (TX100) is a non-ionic surfactant used to permeabilize living cell membranes. The Triton detergent monomer inserts into the lipid membrane disrupting cellular structure and causes permeabilization of the cell membrane [3].

Standard ZN technique

1. Preparation of bacterial smear [2X3cm] on clean and grease free slide, using sterile technique.
2. Air drying [15-30 min] and then heat fixation.
3. 1% Carbolfuchsin staining and heating for 8 min. Washing with clean water.
4. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water.
5. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.
6. Examination of the smear microscopically, using the 100 X oil immersion objective.

New Technique 1 – 0.3%TZN [Triton ZN]

1. Preparation of bacterial smear [2X3cm] on clean and grease free slide, using sterile technique.
2. Air drying [15-30 min] and then heat fixation.
3. Addition of 0.3% Triton [in methanol] and wait for 30 min. washing with clean water.
4. 0.3% Triton in 1% Carbolfuchsin staining and heating for 8 min. washing with clean water.
5. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolorization. Washing with clean water.
6. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.
7. Examination of the smear microscopically, using the 100 X oil immersion objective.

New Technique 2 – 1%TZN

1. Preparation of bacterial smear [2X3cm] on clean and grease free slide, using sterile technique.
2. Air drying [15-30 min] and then heat fixation.
3. Addition of 1% Triton [in methanol] and wait for 30 min. washing with clean water.
4. 0.3% Triton in 1%Carbolfuchsin staining and heating for 8 min. washing with clean water.
5. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water.
6. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.
7. Examination of the smear microscopically, using the 100 X oil immersion objective.

New Technique 3 – 10%TZN

1. Preparation of bacterial smear [2X3cm] on clean and grease free slide, using sterile technique.
2. Air drying [15-30 min] and then heat fixation.
3. Addition of 10% Triton [in methanol] and wait for 30 min. washing with clean water.
4. 0.3% Triton in 1% Carbolfuchsin staining and heating for 8 min. washing with clean water.
5. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water.
6. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.
7. Examination of the smear microscopically, using the 100 X oil immersion objective.

New Technique 4 – 25%TZN

1. Preparation of bacterial smear [2X3cm] on clean and grease free slide, using sterile technique.
2. Air drying [15-30 min] and then heat fixation.
3. Addition of 25% Triton [in methanol] and wait for 30 min. washing with clean water.
4. 0.3% Triton in 1%Carbolfuchsin staining and heating for 8 min. washing with clean water.
5. Addition of 3% v/v hydrochloric acid alcohol for 5 minutes for decolourisation. Washing with clean water.
6. Counter stain with methylene blue for 1–2 minute. Washing with clean water and air drying.
7. Examination of the smear microscopically, using the 100 X oil immersion objective.

Grading of the slides was done by examining 100 fields. For analysis the results were quantified as an AFB score as follows:

Statistics

We compared the mean AFB score by the new technique with conventional ZN technique by Bland – Altman plots. 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 Bland – Altman plots. 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.

Results

The typical smears by the conventional ZN and the four new techniques are shown in the figure 1.

Comparison of ZN and 0.3%TZN by Bland Altman plot [Fig 2]

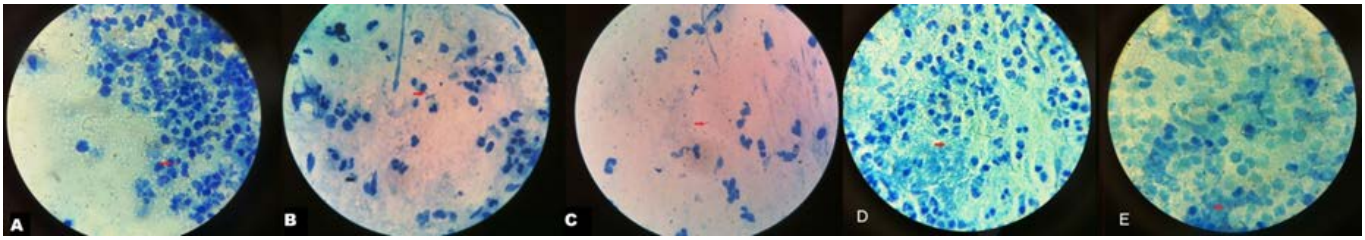

Fig 1: Typical smears by the AFB staining methods. A-Conventional ZN method, B-0.3%Triton ZN method, C-1%Triton ZN method, D-10%Triton ZN method, and E-25%Triton ZN method

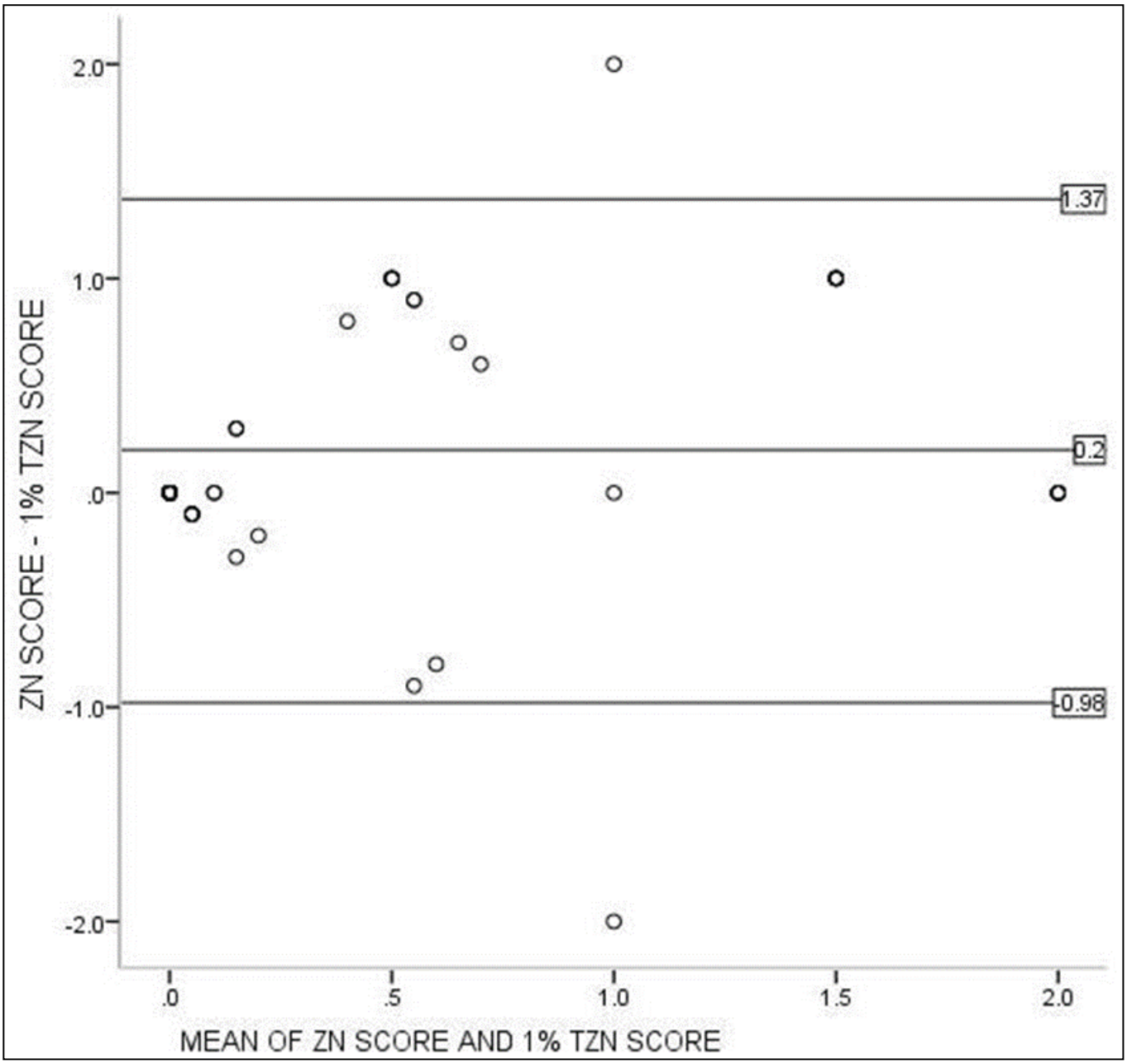

Fig 2: Bland-Altman Plot comparing conventional ZN and 0.3% Triton ZN methods

As there was difference in the two sets of mean AFB score values, they were not same or exactly equal methods. As the mean difference [0.20] of the AFB scores was not zero, the difference in the values was not simply due to imprecise measurements or errors. And conventional ZN technique on the average showed a higher AFB score of 0.2 than 0.3% TZN method.

One sample t test with null hypothesis that mean difference was not significantly away from zero [two methods agree] in this case was done. As p= 0.005, alternate hypothesis was right. Hence the two methods did not agree.

In this case however we wanted to see if the new technique was showing higher AFB score [detection of more number of bacteria]. We used the B & A plot.

The upper limit of agreement was 1.36[0.20+(1.96SD)] and the lower limit was -0.95.

By doing linear regression between the difference set and mean set [p=0.07] there was no significant consistent data above or below the mean difference line.
So the new technique neither agrees with conventional ZN technique nor does it detect higher number of MTB.

ZN and 1%TZN [Fig 3]

The mean difference of AFB score between ZN and 1TZN was 0.20. [Fig 3] The mean difference was statistically significant [p= 0.018]. Hence the two methods do not agree. And conventional ZN technique on the average showed a higher AFB score of 0.2 than 0.3% TZN method.

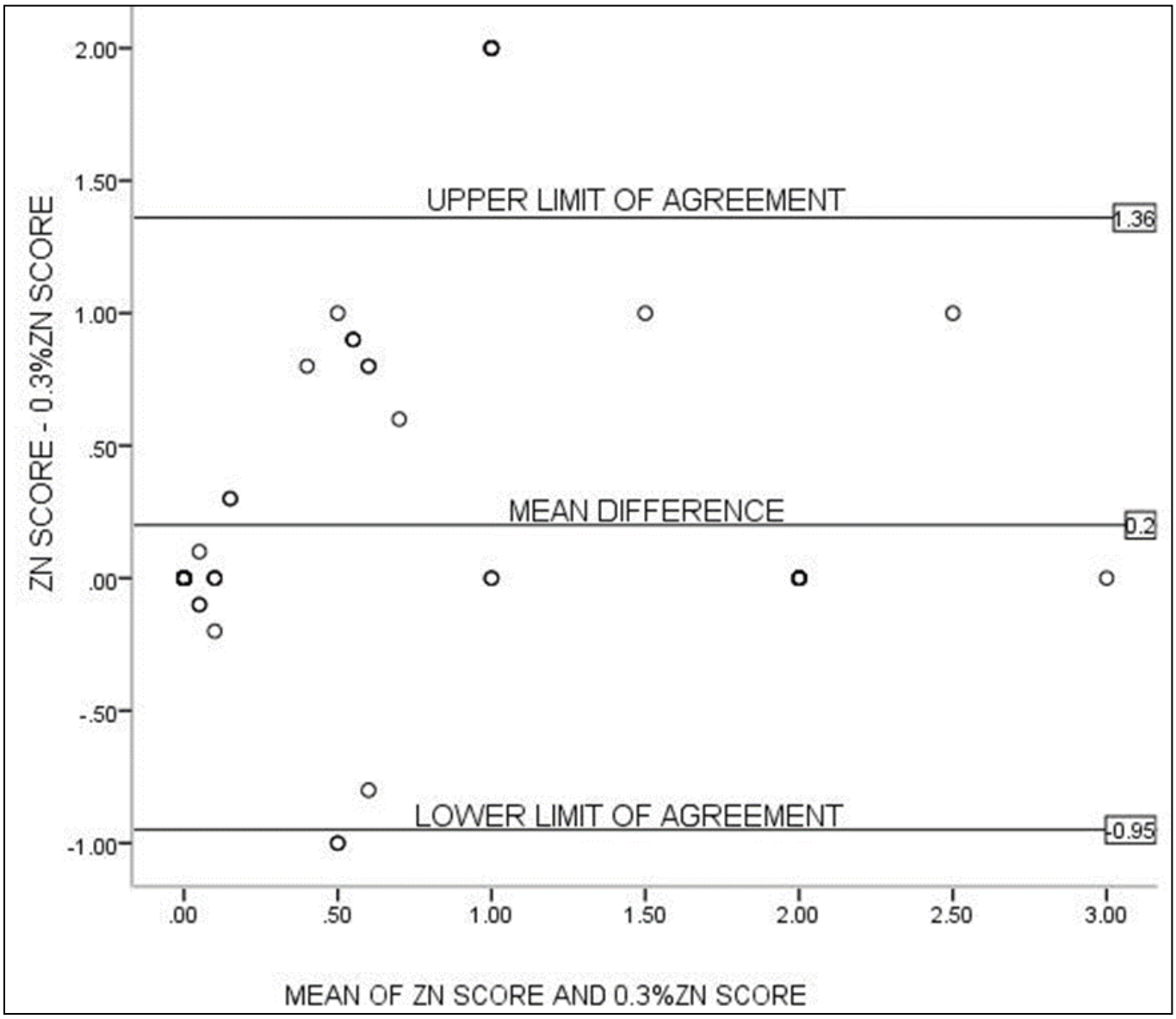

Fig 3: Bland-Altman Plot comparing conventional ZN and 1% Triton ZN methods

The upper limit of agreement was 1.37 and the lower limit was -0.98. Regression between the difference set and mean set was significant [p=0.04].
So the 1% TZN technique detects lower number of MTB than conventional ZN technique.

ZN and 10%TZN [Fig 4]

The mean difference of AFB score between ZN and 1TZN was 0.40. [Fig 3] The mean difference was statistically significant [p< 0.001]. Hence the two methods do not agree. And conventional ZN technique on the average showed a higher AFB score of 0.4 than 0.3% TZN method.

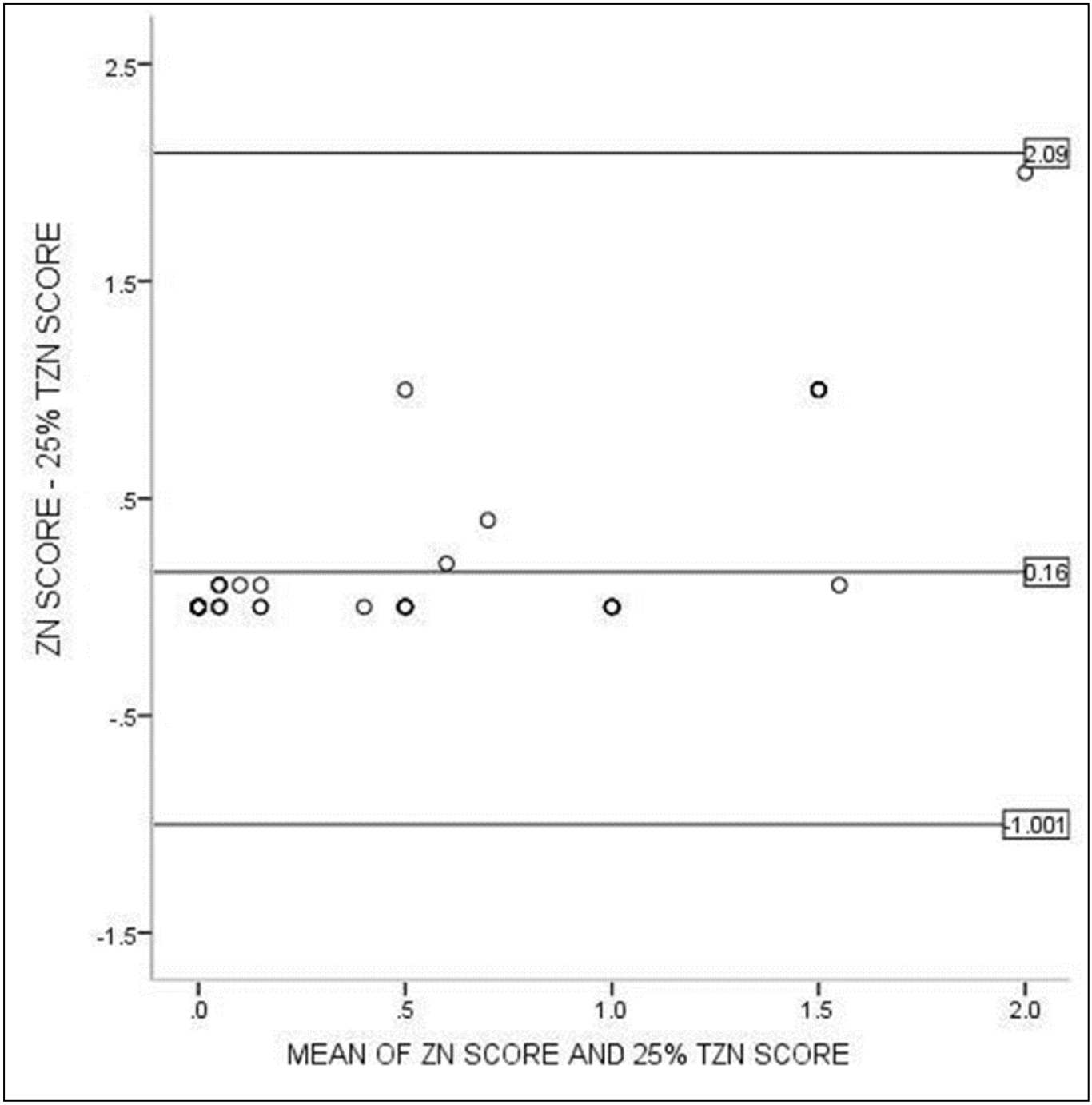

Fig 4: Bland-Altman Plot comparing conventional ZN and 10% Triton ZN methods

The upper limit of agreement was 1.68 and the lower limit was -0.90. Regression between the difference set and mean set was significant [p=0.03].
So the 10% TZN technique do detects lower number of MTB than conventional ZN technique.

ZN and 25%TZN [Fig 5]

The mean difference of AFB score between ZN and 1TZN was 0.54. [Fig 3] The mean difference was statistically significant [p> 0.001]. Hence the two methods do not agree. And conventional ZN technique on the average showed a higher AFB score of 0.5 than 0.3% TZN method.

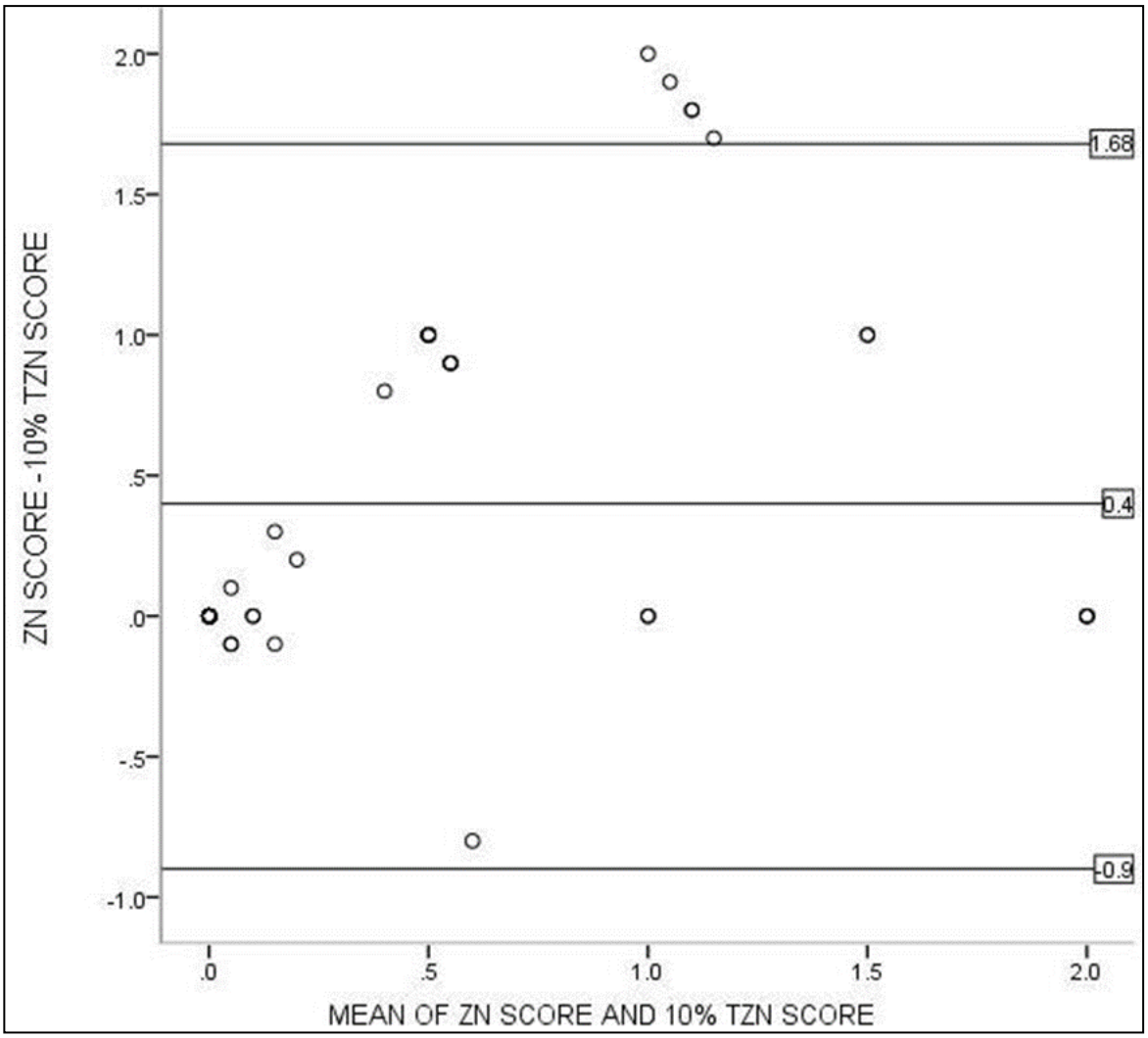

Fig 5: Bland-Altman Plot comparing conventional ZN and 25% Triton ZN methods

The upper limit of agreement was 2.09 and the lower limit was -1.001. Regression between the differences set and mean set was significant [p> 0.001].

So the 25%TZN technique also detects lower number of MTB than conventional ZN technique.

Discussion

We evaluated the performance of Triton permeabilisation modification of ZN staining technique to detect MTB in the sputum of patients with suspected pulmonary tuberculosis. The new technique was inferior to the conventional ZN technique.

One of the reasons for the failure of the Triton addition technique in sputum might be due to the different composition of sputum compared to CSF. The composition of sputum is very different from CSF. It contains variable amounts of carbohydrate, protein, lipids, DNA, α-antitrysin, LDH, lysozyme, lactoferrin and other substances [9]. These may interfere with the actions of Triton.

Chen, *et al.* showed a high bacterial detection rate in CSF with the addition of Triton to ZN staining method to detect AFB. Perhaps using cytocentrifuge and poly L lysine slides might have shown the new technique in better light, but the expense of these additions to the conventional technique would be difficult to implement in resource poor settings.

Experimenting with HeLa cells, Koley *et al.* [3] showed that 0.17 to 0.18mM concentration of TritonX100 for 20 min was ideal to permeabilize the cell membrane. Our work based on Chen, *et al.* [2] Did not show the advantage of triton addition, but a concentration of 0.17 to 0.18mM also needs to be attempted. If the Triton is mixed with carbolfuchsin in one step, the extra washing may be avoided. This might prevent the loss of cells and bacilli. The small sample size is a limitation of our study.

Pending these experiments, our study shows that addition Triton dye to ZN staining technique may not improve the detection of MTB in sputum.

Table 1: Scoring system used for analysis

Observation	RNTCP Grading	AFB Score
No AFB in 300 fields	Zero	0
Less than 10 AFB in 100 fields	Scanty [no of AFB seen are recorded]	0.X
10 to 99 AFB in 100 fields	One plus	1
1- 10 AFB in one field	Two plus	2
More than 10 AFB in one field	Three plus	3

X=no of AFB seen

Acknowledgements
We thank Dr Ravi Kiran for his help with microscopy.

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