Sensitivity of Ziehl-Neelsen method for centrifuged deposit smears of sputum samples transported in cetyl-pyridinium chloride

N. Selvakumar, M. Gomathi Sekar, Vanaja Kumar, D. Vijaya Bhaskar Rao, Fathima Rahman & P.R. Narayanan

Tuberculosis Research Centre (ICMR), Chennai, India

Received August 8, 2005

Background & objectives: Sensitivity of Ziehl-Neelsen (ZN) method is known to be low for liquefied sputum smears. Information on the ZN sensitivity for centrifuged deposit smears is not known. This study was carried out to determine the sensitivity of ZN method for acid fast bacilli (AFB) in centrifuged deposit smears and liquefied sputum smears made from sputum samples transported in cetyl-pyridinium chloride (CPC) solution.

Methods: Liquefied sputum smears and the corresponding centrifuged deposit smears from each of the 607 consecutive sputum samples collected from tuberculosis patients admitted to receive treatment transported in CPC were read by the same readers and their results compared with culture results.

Results: A significantly (P<0.001) higher proportion of samples were positive in centrifuged deposit smears (40%) compared to liquefied sputum smears (30%). The results of 341 culture-positive specimens revealed that the sensitivity of ZN method was 47 per cent using liquefied sputum smears and 63 per cent using centrifuged deposit smears (P<0.001).

Interpretation & conclusion: Our study demonstrated that the sensitivity of ZN method for AFB in centrifuged deposit smears and liquefied sputum smears was reduced if sputum samples are transported in CPC solution.

Key words Acid fast bacilli - cetyl-pyridinium chloride - Mycobacterium tuberculosis - Ziehl-Neelsen staining
determine the sensitivity of ZN method for acid fast bacilli (AFB) in liquefied sputum smears and for centrifuged deposit smears prepared from sputum samples transported in CPC solution.

Material & Methods

Sputum samples and transportation: In an ongoing study implementing Revised National Tuberculosis Control Programme (RNTCP) regimens in a Tuberculosis Unit, Tiruvallur district, Tamil Nadu, India, 607 samples collected consecutively between July 2003 and March 2004 were sent to Tuberculosis Research Centre (TRC), Chennai, for bacteriological examination. The samples were collected from patients admitted to receive RNTCP regimens and also from patients who were followed up during treatment. The CPC solution was prepared in TRC and distributed in sterile bottles to health facilities for collection of sputum. The patients were instructed to collect sputum in 5 ml of CPC solution in universal containers3. The samples were kept under ambient conditions in the health facilities till they were transported to TRC. The delay between the collection and processing the specimens for culture varied from 4 to 10 days.

Liquefied sputum smears and the centrifuged deposit smears: Soon after receiving the samples in TRC, smears (liquefied sputum smears) were prepared from each of the liquefied sample. The sputum samples were then centrifuged at 3000 g for 15 min and the deposits were re-suspended in 20 ml of sterile distilled water. The samples were again centrifuged as before and smears were made from the deposits (centrifuged deposit smear). Culture was set up for all the deposits for M. tuberculosis3.

Preparation of ZN staining reagents: Chemicals: Ethanol, phenol, sulphuric acid, methylene blue from Qualigens, Mumbai, India; Basic-fuchsin from Hi-Media, Mumbai, India, were used in the study.

Reagents: 1 per cent carbol-fuchsin: 10 g of basic-fuchsin was made to dissolve in 100 ml of ethanol and 50 ml of molten phenol in a flask maintained at 60°C in a water-bath. This solution was made up to 1000 ml with distilled water.

Sulphuric acid (25%): 250 ml of concentrated sulphuric acid was slowly added to 750 ml of distilled water.

Methylene blue (0.1%): 1 g of methylene blue was dissolved in 1000 ml of distilled water.

ZN staining method: The RNTCP guidelines were followed for the staining, examining and grading of direct smears4. The air-dried smear slides were fixed over a flame 3-5 times for 3-4 seconds. The slides were then placed on a staining rack and filtered carbol-fuchsins was poured over to cover the entire slide. The slides were heated from underneath until vapours started rising. After 5 min, slides were gently rinsed with tap water to remove the excess carbol-fuchsins stain. The smears were decolourised with 25 per cent sulphuric acid for 2-4 min and again rinsed with water. The slides were counterstained for 30 seconds using 0.1 per cent methylene blue solution. The slides were rinsed with tap water, allowed to dry, and examine under a binocular microscope.

Quality of sputum AFB microscopy: After staining, the slides were coded by the statisticians. The same technician read the liquefied sputum smears and the corresponding centrifuged deposit smears. A senior technician crosschecked 20 per cent of the systematically selected smears (144 each of liquefied and deposit smears) in a blinded fashion and an umpire resolved the discrepant smears. The results were decoded and matched for comparison. In addition, from every tenth sample, one extra liquefied sputum smear and centrifuged deposit smear were made (56 in each) and stained by ZN method.

Statistical analysis: Smear and culture results were entered in Microsoft Excel sheets and SPSS/PC+, version 4.0 (SPSS Inc. Chicago, IL, 1990) package was used for analysis. The significance of the observed difference between the staining methods was determined using McNemar’s test. P < 0.05 were considered as significant. Kappa statistics were used
to know the significance of agreement in reading duplicate smears.

Results & Discussion

*Comparison of liquefied sputum smears and centrifuged deposit smears*: The ZN method yielded 30 (185 of 607) and 40 per cent (245 of 607) AFB positives respectively using liquefied sputum and centrifuged deposit smears ($P < 0.001$).

Of the 607 samples cultured, 341, 17 and 7 yielded *M. tuberculosis*, contaminants and non-tuberculous mycobacteria (NTM), respectively, and the remaining 242 were negative for culture. After excluding the contaminants and NTM, 583 specimens were considered for analysis. The sensitivity and specificity of ZN method against culture as the gold standard was 63 and 47, and 94 and 95 per cent, respectively for centrifuged deposit and liquefied sputum smears (Table).

*Quality assurance (QA) of smear microscopy*: The Kappa value of 0.80 and 0.81, respectively for each of 56 duplicate liquefied and centrifuged deposit smears ensured the reproducibility of reading the smears. Only 1 false negative and 1 false positive error made in each of 144 liquefied and centrifuged deposit smears ensured the quality of reading.

In our earlier study using ZN method, of the 104 sputum samples stored in CPC solution, 52 were positive for AFB by direct sputum smears compared to 35 (67.3%) by liquefied sputum smears$^5$. In another study, of the 85 sputum samples stored in CPC solution, 98 per cent were AFB positive by direct sputum smears compared to 38 per cent in centrifuged deposit smears where the deposits were obtained after the samples were processed by N-acetyl L-cysteine - sodium hydroxide method$^6$. Both the studies have shown significant loss of smear positivity either using the liquefied sputum smears or the centrifuged deposit smears. In the present study, the centrifuged deposit of sputum sample transported in CPC solution was washed with distilled water and the deposit obtained after re-centrifugation was used for making smear$^3$.

Our experience in TRC$^5$ revealed that the sensitivity of ZN method for liquefied sputum smears was 47 per cent. The observed low sensitivity of the ZN method for liquefied sputum smears could have been due to the dilution of the sample and the dilution varied from 1 to 4 times depending on the volume of sputum collected in 5 ml of CPC solution.

The present study determined the sensitivity of the ZN method for the centrifuged deposit smears. The observed sensitivity of 63 per cent for the centrifuged deposit smears in the present study was almost similar to the sensitivity reported for direct sputum smears (47 to 65%)$^7$. The failure to achieve the improved sensitivity for centrifuged deposit smears could be attributed to the exposure of sputum samples to CPC, as the physical and chemical injury to the integrity of the cell wall causes the AFB to lose their acid-fastness$^8$. Cetrimide, a quaternary ammonium salt,

<table>
<thead>
<tr>
<th>Culture result</th>
<th>Liquefied sputum smear</th>
<th>Centrifuged deposit smear</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>159</td>
<td>182</td>
<td>216</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>230</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>171</td>
<td>412</td>
<td>230</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>47</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>95</td>
<td></td>
<td>94</td>
</tr>
</tbody>
</table>
disrupts the proton motive force of the bacterial cell membrane required for solute transport and generation of adenosine tri-phosphate at the cell membrane\textsuperscript{9} and CPC, another quaternary ammonium salt, interferes with the fluorescence staining of Nocardia asteroids, an acid-fast organism\textsuperscript{10}.

The reduced specificity (94\%) could be due to the inclusion of sputum samples collected from patients receiving treatment as 20 to 25 per cent of the samples collected from patients receiving rifampicin containing regimens are reported to be smear positive but culture negative\textsuperscript{11}.

The limitation of the study is that the sensitivity of the ZN method for direct sputum smears could not be determined as these specimens were collected from the peripheral health institutions and transported to TRC for culture of \textit{M. tuberculosis}.

In conclusion, the sensitivity of ZN method for centrifuged deposit smears and liquefied sputum smears was found to be reduced if sputum samples were transported in CPC solution.

Acknowledgment

The authors are grateful to all the medical officers of Velliuyr Tuberculosis Unit, Tiruullur district, Tamil Nadu, India, for their support in the collection of sputum samples from the patients; the staff of epidemiology unit for transporting the samples; B. Mahizhaveni, A. Radhakrishnan, S. Sudhamathi, J. Samuvel Vasathan and staff of the Department of Bacteriology for their technical support; Dr. Armand Van Deun, Consultant Microbiologist, International Union Against Tuberculosis and Lung Disease, Paris, France for his critical review of this manuscript. The study was supported in part by the WHO, with funds from USAID, through SEARO, New Delhi.

References


Reprint requests: Dr N. Selvakumar, Deputy Director, (IUATLD-Regional Advisor)
Tuberculosis Research Centre (ICMR), Chetput, Chennai, 600 031, India
e-mail: selvakumarn@hotmail.com