A Highly Efficient Ziehl-Neelsen Stain: Identifying De Novo Intracellular *Mycobacterium tuberculosis* and Improving Detection of Extracellular *M. tuberculosis* in Cerebrospinal Fluid

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Tuberculous meningitis leads to a devastating outcome, and early diagnosis and rapid chemotherapy are vital to reduce morbidity and mortality. Since *Mycobacterium tuberculosis* is a kind of cytozoic pathogen and its numbers are very few in cerebrospinal fluid, detecting *M. tuberculosis* in cerebrospinal fluid from tuberculous meningitis patients is still a challenge for clinicians. Ziehl-Neelsen stain, the current feasible microbiological method for the diagnosis of tuberculosis, often needs a large amount of cerebrospinal fluid specimen but shows a low detection rate of *M. tuberculosis*. Here, we developed a modified Ziehl-Neelsen stain, involving cytopsin slides with Triton processing, in which only 0.5 ml of cerebrospinal fluid specimens was required. This method not only improved the detection rate of extracellular *M. tuberculosis* significantly but also identified intracellular *M. tuberculosis* in the neutrophils, monocytes, and lymphocytes clearly. Thus, our modified method is more effective and sensitive than the conventional Ziehl-Neelsen stain, providing clinicians a convenient yet powerful tool for rapidly diagnosing tuberculous meningitis.

**T**uberculous meningitis (TBM) is the most severe form of tuberculosis and causes substantial morbidity and mortality (18). The early diagnosis of and prompt initiation of chemotherapy for TBM are crucial to a successful outcome. However, the early and accurate detection of *Mycobacterium tuberculosis* in the cerebrospinal fluid (CSF) of TBM patients still remains a challenge for clinicians, mainly due to the lack of rapid, efficient, and practical detection methods (30).

Currently, mycobacterial culture is the gold standard for detecting *M. tuberculosis*, but it is time-consuming and requires specialized safety procedures in laboratories (19, 26). Serological methods are convenient but lack sensitivity and specificity (4, 7). Although the PCR technique is rapid, it is costly for routine use in developing countries where most tuberculosis cases occur (5, 17, 21, 24). Conventional smear microscopy with the Ziehl-Neelsen (ZN) stain is a rapid and practical method for detecting acid-fast bacilli (AFB), especially in low-income countries, due to its rapidity, low cost, and high positive predictive value for tuberculosis (14). However, the Ziehl-Neelsen method is severely handicapped by its low detection rate, ranging from 0 to 20% for CSF specimens (31–33). One of the main reasons behind this is that *M. tuberculosis* can hardly be stained by acid-fast dyes once it enters the cells. Another important reason is that the Ziehl-Neelsen method requires a large volume of CSF for TBM diagnosis, as it is incapable of detecting bacilli that are fewer than 10,000 in number per slide or per ml of specimen (32, 33). Therefore, it is important to develop an alternative, cost-effective method for detecting intracellular *M. tuberculosis*. Additionally, knowing which cell type is infected by *M. tuberculosis* in the CSF of TBM patients could help us to unravel new antituberculous candidates (10).

To reveal the presence of intracellular *M. tuberculosis* and improve the detection of extracellular *M. tuberculosis* from a small volume of CSF specimens, we developed a highly efficient Ziehl-Neelsen stain involving the use of only 0.5-ml CSF specimens from TBM cases. The formed elements of the CSF, including the bacilli and cells, were compactly collected onto the slides by cytopsinning followed by staining with acid-fast dyes containing the detergent Triton X-100. Using this modified staining method, AFB can be clearly revealed within the immune cells, and the detection rate of extracellular AFB was significantly improved as well.

**MATERIALS AND METHODS**

**Study subjects.** The study protocol was approved by the Institutional Review Board of Xijing Hospital, the Fourth Military Medical University, Xian, China, and written informed consent was obtained from all patients or their legal surrogates. Twenty-nine patients whose CSF specimens were culture positive for *Mycobacterium* by the MGIT 960 mycobacteria culture system were diagnosed with TBM and enrolled in this study. For the identification of *M. tuberculosis* and differentiation of non-*M. tuberculosis* bacteria from positive cultures, the Ziehl-Neelsen stain and a commercial TB real-time PCR kit (Qiagen GmBH, Hilden, Germany) were used.

**Conventional Ziehl-Neelsen stain.** Two-ml CSF specimens were centrifuged at 3,000 × g for 15 min, and the sediment was smeared on slides as previously described (27). All smears were stained by the conventional Ziehl-Neelsen method for the presence of AFB as depicted earlier (14) and observed under a light microscope.

**Modified Ziehl-Neelsen stain.** Cytopsin was used to collect the formed elements of CSF specimens. In brief, 0.5-ml CSF specimens were...
Forty-eight CSF samples were collected from 29 TBM patients. ZN, Ziehl-Neelsen stain was performed on each sample (n = 48) and patient (n = 29). The specificity of all techniques was 100% (95% CI, 66.51 to 79.33) compared to those of culture and the modified Ziehl-Neelsen stain. The sensitivity of all techniques analyzed by sample (48/48) and patient (29) was 100% (29/29). These data suggest a higher sensitivity of our modified Ziehl-Neelsen stain than those of mycobacterial culture and the conventional Ziehl-Neelsen stain.

In the conventional method, a relatively large CSF volume (2 ml) was used for smear slides. Ziehl-Neelsen stain showed that the AFB were sparsely distributed and the integrity of immune cells was violated (Fig. 1A). In some regions of smear slides, aggregated cells often were observed in AFB-positive fields (Fig. 1B). We noted that in the conventional method, all of the AFB observed were distributed extracellularly and no AFB were found within the cells. In the modified method, 0.5-ml CSF samples were used for cytospin slides. Ziehl-Neelsen stain with Triton processing showed that the immune cells were distributed evenly on the slides and maintained the integrity of cellular morphology (Fig. 1C to F). Moreover, using this method, we clearly observed AFB within the immune cells, including neutrophils, monocytes, and lymphocytes (Fig. 1D to F). Importantly, we also found that the number of extracellular AFB was significantly increased in the modified method. In addition, we noted that cells containing AFB displayed an abnormal morphology relative to those free of AFB. For example, a larger percentage of neutrophils with more lobes showed an obvious right shift (Fig. 1D, arrow) and monocytes displayed a larger cell body and foam-like cytoplasm (Fig. 1E, arrow), while lymphocytes showed a smaller cell body and nuclear pyknosis (Fig. 1F, arrow). These data suggest that upon and following phagocytosis by the immune cells, intracellular M. tuberculosis also exerts an effect on the immune cells.

To confirm the intracellular localization of AFB and exclude the possibility that the bacilli inadvertently adhere to the cell sur-

**TABLE 1** Sensitivity of all techniques analyzed by sample (n = 48) and patient (n = 29)*

<table>
<thead>
<tr>
<th>Analysis type (n)</th>
<th>% Sensitivity (no. of positive samples) of each technique</th>
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<tbody>
<tr>
<td>Patient (29)</td>
<td>Culture ZN stain Modified ZN stain</td>
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<tr>
<td></td>
<td>100 (29) 27.6 (8) 100 (29)</td>
</tr>
<tr>
<td>Sample (48)</td>
<td>72.9 (35) 16.7 (8) 100 (48)</td>
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*Forty-eight CSF samples were collected from 29 TBM patients. ZN, Ziehl-Neelsen.

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We performed the double labeling of AO, a fluorescent dye used for staining tubercle bacilli (28, 31), the activated neutrophil marker CD11b, monocyte marker ED1, and T lymphocyte marker CD3 or B lymphocyte marker CD20. Our results showed that AO l Bacilli were indeed located in the cytoplasm of neutrophils, monocytes, or lymphocytes, but not on the surface of these cells (Fig. 2 and 3).

For the quantitative analysis of extracellular AFB, the conventional method identified extracellular AFB in 8 of 48 CSF specimens (16.7%), while the modified method identified all of the specimens (100%; 48/48). For the quantification of intracellular AFB, the modified method detected intracellular AFB in 45 of 48 CSF specimens (93.8%), while none was detected by the conventional method (Table 2). Furthermore, through the observation of 300 fields on each slide from 48 CSF samples, we found that the number of extracellular and intracellular AFB-positive fields with the conventional method was 20.0 ± 63.1 and 0 fields, respectively, and 73.5 ± 58.0 (P < 0.001) and 24.3 ± 22.0 (P < 0.001) fields with the modified method, respectively (Fig. 4).

**DISCUSSION**

In the present study, we developed a modified Ziehl-Neelsen method on cytospin slides with Triton processing. Only using small CSF samples, our modified method yielded a 93.8% detection rate of intracellular AFB, while the conventional method failed to detect any intracellular AFB. Also, this method can improve the detection rate of extracellular AFB from 16.7% of that of the conventional Ziehl-Neelsen stain to 100%. Moreover, our method had a higher sensitivity than the mycobacterium culture method and is easier to implement than AO staining, in which expensive fluorescence microscopy is required.

We attributed the higher detection rate of M. tuberculosis by the modified method to two reasons. First, cytospinning was employed, by which AFB within cells were concentrated compactly in a small circular area on the slide with low-speed centrifugation. In contrast to the conventional method, in which the cells are mostly destroyed, our modified method preserves the cellular integrity and consequently prevents the loss of intracellular AFB from inside the cells. In addition, coating the slides with poly-L-lysine further prevents cells and bacilli in the CSF from falling off during staining. Second, we employed Triton X-100, which permeates the cellular membrane and facilitates the entry of acid-fast dye. Thus, the modified method combining cytospin and Triton permeation significantly improves the efficiency of the Ziehl-Neelsen stain. Furthermore, the cytospin method concentrates extracellular AFB in the CSF. Consequently, the CSF specimens from all 48 specimens were positive for extracellular AFB by the modified stain, whereas the conventional method had a low detection rate (16.7%; 8/48). Triton processing also improves the permeability...
of the unique bacterial wall of AFB (15), which resists staining by acid-fast dyes. Thus, the modified method also improved the detection rate of extracellular AFB.

Accumulating evidence shows that the presence of intracellular \textit{M. tuberculosis} is a crucial indicator of the body’s immune response to tuberculosis (26, 28). Monocytes, neutrophils, and lymphocytes are three major immune cell types in the CSF of TBM, and they play different roles in the pathogenesis of TBM. Monocytes are regarded as the main immune cells for host defense against \textit{M. tuberculosis}. However, a group of studies have shown that the antigen-presenting function of macrophages is significantly impaired following infection with \textit{M. tuberculosis} (9, 12, 20, 22). Thus, it is proposed that other immune cells can be recruited to enhance the immune response against \textit{M. tuberculosis} infection (16). In the present study, we revealed that \textit{M. tuberculosis} was present in both neutrophils and lymphocytes, indicating that these two types of cells are indeed involved in the host’s immune response against \textit{M. tuberculosis}. Neutrophils have both bactericidal and immunomodulatory functions (3, 13, 25). When infected with \textit{M. tuberculosis}, neutrophils can directly kill invading \textit{M. tuberculosis} via the generation of reactive oxygen species and the release of preformed oxidants and proteolytic enzymes, and/or indirectly eliminate \textit{M. tuberculosis} by releasing an array of cytokines and chemokines to attract other inflammatory cells. On the other hand, infected neutrophils also provide a permissive site for the active replication of \textit{M. tuberculosis} (6, 8), which in turn induces the apoptosis of neutrophils (1, 2). Macrophages are capable of phagocytizing apoptotic neutrophils, resulting in the eventual elimination of intracellular \textit{M. tuberculosis} (29). Unexpectedly, our results also showed an intracellular distribution of \textit{M. tuberculosis} in CD3$^+$ T lymphocytes. Previous studies have shown that dendritic cells, a derivative from the lymphocyte precursor, can phagocytize \textit{M. tuberculosis} and exert their antigen-presenting functions (11, 23). These findings together prompt us to propose that lymphocytes play a similar role in TBM, compensating for the decreased antigen-presenting function of macrophages. Thus, our present study suggests that in addition to monocytes, neutrophils and lymphocytes also participate in the host defense against \textit{M. tuberculosis} infection.

\textbf{Conclusions.} Given the acute need for a simple, efficient, and practical method for detecting \textit{M. tuberculosis} within the cells and from small CSF samples, our modified Ziehl-Neelsen staining method can efficiently reveal the presence of AFB in the immune cells of small CSF samples from TBM patients and improve the

\begin{table}
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\begin{tabular}{|l|c|c|}
\hline
ABF group (n) & \% Positive rate (no. of positive samples) & \% Positive rate (no. of positive samples) \\
& & of each technique \\
\hline
Extracellular (48) & 16.7 (8) & 100 (48) \\
Intracellular (48) & 0 (0) & 93.8 (45) \\
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\end{tabular}
\caption{Positive rate of intracellular and extracellular ABF in the CSF samples (n = 48) detected by ZN stain and modified ZN stain}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Intracellular distribution of AFB in lymphocytes on the modified Ziehl-Neelsen stain. Double labeling of AO (A and D, green) with CD3 (B, red) and CD20 (E, red) shows the intracellular location of AFB in lymphocytes (C and F). The nuclei are stained by Hoechst 33342 (blue). Scale bar, 5 \textmu m.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure4}
\caption{Comparison of AFB-positive fields by the conventional (CZN) and modified (MZN) Ziehl-Neelsen stain. Three hundred fields on each slide from 48 CSF specimens were observed. Compared to the ZN stain, which reveals no intracellular AFB-positive fields, the modified ZN stain definitively identifies AFB within the immune cells. Moreover, the modified stain reveals more extracellular AFB-positive fields than the conventional ZN stain.}
\end{figure}
detection rate of extracellular AFB as well. Therefore, this method will be of tremendous value in improving both the diagnosis and treatment of tuberculosis.

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