Diagnostic Accuracy of Intracellular *Mycobacterium tuberculosis* Detection for Tuberculous Meningitis

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Abstract

Rationale: Early diagnosis and treatment of tuberculous meningitis saves lives, but current laboratory diagnostic tests lack sensitivity.

Objectives: We investigated whether the detection of intracellular bacteria by a modified Ziehl-Neelsen stain and early secretory antigen target (ESAT)-6 in cerebrospinal fluid leukocytes improves tuberculous meningitis diagnosis.

Methods: Cerebrospinal fluid specimens from patients with suspected tuberculous meningitis were stained by conventional Ziehl-Neelsen stain, a modified Ziehl-Neelsen stain involving cytospin slides with Triton processing, and an ESAT-6 immunocytochemical stain. Acid-fast bacteria and ESAT-6-expressing leukocytes were detected by microscopy. All tests were performed prospectively in a central laboratory by experienced technicians masked to the patients' final diagnosis.

Measurements and Main Results: Two hundred and eighty patients with suspected tuberculous meningitis were enrolled.

Thirty-seven had *Mycobacterium tuberculosis* cultured from cerebrospinal fluid; 40 had a microbiologically confirmed alternative diagnosis; the rest had probable or possible tuberculous meningitis according to published criteria. Against a clinical diagnostic gold standard the sensitivity of conventional Ziehl-Neelsen stain was 3.3% (95% confidence interval, 1.6–6.7%), compared with 82.9% (95% confidence interval, 77.4–87.3%) for modified Ziehl-Neelsen stain and 75.1% (95% confidence interval, 68.8–80.6%) for ESAT-6 immunostain. Intracellular bacteria were seen in 87.8% of the slides positive by the modified Ziehl-Neelsen stain. The specificity of modified Ziehl-Neelsen and ESAT-6 stain was 85.0% (95% confidence interval, 69.4–93.8%) and 90.0% (95% confidence interval, 75.4–96.7%), respectively.

Conclusions: Enhanced bacterial detection by simple modification of the Ziehl-Neelsen stain and an ESAT-6 intracellular stain improve the laboratory diagnosis of tuberculous meningitis.

Keywords: tuberculosis; central nervous system; cerebrospinal fluid; diagnosis

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At a Glance Commentary

Scientific Knowledge on the

Subject: Tuberculous meningitis is the most lethal form of tuberculosis, but current laboratory diagnostic tests are inadequate, and delayed diagnosis and treatment is common.

What This Study Adds to the Field:

We describe two new diagnostic tests that respectively enhance the detection of *Mycobacterium tuberculosis* and one of its antigens (early secreted antigen target-6) in cerebrospinal fluid leucocytes. Both tests are substantially more sensitive than conventional Ziehl-Neelsen stain of cerebrospinal fluid. In particular, simple modifications to the Ziehl-Neelsen stain could be widely and rapidly adopted by laboratories worldwide and might dramatically improve the diagnosis of this lethal infection.

Tuberculous meningitis (TBM) is the most lethal form of tuberculosis, killing or maiming around half of sufferers. The likelihood of surviving the infection is greatly increased if antituberculosis chemotherapy is started early, before the onset of coma (1). Early diagnosis, however, is notoriously difficult. Presenting clinical features are nonspecific and all currently available laboratory diagnostic tests lack sensitivity (2). Consequently, diagnostic and treatment delay is common and many either die or are left neurologically disabled.

The 130-year-old Ziehl-Neelsen (ZN) stain of cerebrospinal fluid (CSF) remains the cornerstone of TBM diagnosis in most settings, but the performance of light microscopy to detect acid-fast bacilli (AFB) in CSF is highly variable and often extremely poor. The sensitivity can be substantially improved by increasing the volume of CSF examined and meticulous microscopy (3), but even in the best hands sensitivity rarely exceeds 60% (4). The detection of *Mycobacterium tuberculosis* nucleic acid in CSF by nucleic acid amplification (e.g., polymerase chain reaction [PCR]) is a widely studied alternative. These assays can “rule-in” the diagnosis of TBM (i.e., they are highly specific), but limited sensitivity ($\leq 60\%$) means they cannot “rule-out” the

diagnosis (5). The new Xpert MTB/RIF test (Cepheid, Sunnyvale, CA), which uses real-time PCR within an automated cartridge to detect *M. tuberculosis* DNA and mutations conferring rifampicin resistance, has greatly advanced the diagnosis of pulmonary tuberculosis (6), and has recently been evaluated for the diagnosis of TBM. Two large studies reported the sensitivity of Xpert MTB/RIF on CSF was 59.3% (7) and 62% (8), respectively, which is still inadequate. In short, there remains an urgent need to develop new diagnostic tests for TBM that are cheap, rapid, and easy to perform, and improve on the sensitivity of microscopy, culture, and nucleic acid amplification, while retaining high specificity.

We have developed two such novel diagnostic tests. The first is based on the hypothesis that intracellular *M. tuberculosis* may be missed by conventional ZN stain and their detection may be enhanced by improving the presentation of cells on the slide and increasing the permeability of the cells to the stain (modified ZN [MZN] stain). A proof-of-concept study found it revealed AFB by microscopy in 48 of 48 CSF specimens taken from 29 patients treated for TBM as opposed to 8 of 48 positive by conventional ZN stain (9). The second test is based on the premise that the CSF white cells, particularly professional phagocytic cells, of patients with TBM may contain concentrations of *M. tuberculosis*-specific antigens, in particular, early secreted antigen target (ESAT)-6. Others have suggested the detection of CSF ESAT-6 by ELISA might be a good basis for a diagnostic test (10), but intracellular staining for this antigen has not been described previously. Therefore, the aim of the current study was to define the role of the MZN stain and a novel ESAT-6 immunocytochemical stain for the diagnosis of TBM in a large population of Chinese patients with clinically suspected TBM.

Methods

Study Design

We performed a prospective multicenter study comparing conventional ZN stain and mycobacterial culture against MZN and ESAT-6 immunocytochemical stain for the diagnosis of TBM in children and adults. From July 2010 through June 2011, consecutive patients with meningitis were screened for inclusion in the study from 17

hospitals in Shaanxi province, China. The hospitals were of three types: 3,000-bed hospitals providing secondary and tertiary care ($n = 4$); 500–1,500-bed hospitals that serve the local community ($n = 11$); and specialist tertiary referral hospitals for severe tuberculosis ($n = 2$).

Patients were eligible to enter the study as long as they met initial diagnostic criteria for possible, probable, or definite TBM. Published, international TBM diagnostic criteria were applied, which designated a score to patients based on the results of clinical, radiologic, and CSF examination (11). All patients with a diagnostic score of more than five at presentation were eligible to enter the study and their written informed consent to participate was sought. The same criteria were used to define a final diagnosis for each patient, based on the results of further investigations performed while an in-patient. Briefly, TBM was defined as “definite” if AFB were detected by conventional ZN staining of CSF or *M. tuberculosis* cultured from the CSF. “Probable” TBM was defined by a score of more than 9 when cerebral imaging was unavailable, or more than 11 if cerebral imaging was available. “Possible” TBM was defined by a score of 6–9 points if cerebral imaging unavailable or 6–11 points if imaging was available. Patients were excluded if they were infected with HIV, their initial diagnostic score was less than six, or if consent to participate was not obtained.

The case records and CSF specimens of participants were sent to Xijing Hospital, where all tests were performed immediately by four experienced technicians masked to the patients’ diagnoses. The technicians did not have access to the case records, which were used to extract the clinical data. Standard CSF biochemical and microbiologic tests were performed on all specimens, including Gram stain, bacterial culture, and India ink stain. All patients were tested for HIV-specific antibodies (Roche Elecsys HIV Combi, Mannheim, Germany).

The study protocol was approved by the Ethics Committee of the First Attached Hospital of the Fourth Military Medical University and written informed consent was obtained from all participants or their legal surrogates.

Conventional ZN Stain and Mycobacterial Culture

Conventional ZN stain and microscopy and mycobacterial culture in MGIT 960

Mycobacteria Culture System (Becton, Dickinson and Co., Franklin Lakes, NJ) were performed on all CSF specimens (4). Briefly, 2 ml of CSF was spun at $3,000 \times g$ for 10 minutes, and two drops of the deposit dried on slides before staining with carbolfuchsin, decolorization with acidified alcohol, and counterstaining with methylene blue. A total of 300 fields from each slide were examined for AFB by light microscope and the number of positive fields recorded; the numbers of intracellular or extracellular bacteria were not recorded. Cultured *M. tuberculosis* was identified using a PCR-Fluorescence Detection (Da'an Comp, Guangzhou, China) (12). The detected target gene of this kit is the *M. tuberculosis* repetitive sequence IS986. The sequences of the primers and probe are 5'-TCGCCCGTCTACTTGGTGTT-3', 5'-TGATGTGGTCGTAGTAGGTC-3, and 5'-ACAACGCCGAATTGCGAAGGGC-3 (labeled with FAM at the 5' end and with DABCYL at the 3' end).

MZN Staining

The MZN stain was performed on all CSF specimens as described previously (9). In brief, 0.5 ml of CSF was loaded into a cytospin chamber with poly-L-lysine-coated slides and centrifuged at $100 \times g$ for 5 minutes. The slide was fixed with 4% paraformaldehyde for 15 minutes at room temperature then permeabilized with 0.3% TritonX-100 for 30 minutes, before staining with carbolfuchsin containing 0.3% TritonX-100 and counterstained with methylene blue. Three hundred fields of each slide were examined by light microscopy and the numbers of fields and the type of leukocytes (neutrophils, lymphocytes, or monocytes) in which AFB were seen were documented. The numbers of extracellular bacteria were not recorded. Positive slides were confirmed by independent review from another technician and 25% of slides were randomly selected for rereading.

ESAT-6 Immunocytochemical Stain

CSF cytospin slides were prepared as described for the MZN stain, and then subjected to 4% paraformaldehyde fixation at room temperature for 15 minutes. After blocking endogenous peroxidase by incubating in 3% H_2O_2 in phosphate-buffered saline for 10–30 minutes, the slides were incubated with rabbit anti-ESAT-6 polyclonal antibody (Abcam, Cambridge,

MA) at 4°C overnight, followed by incubation with biotinylated antirabbit IgG for 2 hours at room temperature. Next, the slides were incubated with avidin-biotin-peroxidase complex (1:300, Vector, Burlingame, CA) for 2 hours followed by 0.1% 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) and 0.05% H_2O_2 in phosphate-buffered saline for 5–10 minutes at room temperature. Finally, the slides were counterstained with methylene blue and examined by light microscopy. Positive staining was defined as visualization of yellow brown granules in the cytoplasm of cells. The specificity of immune-labeling was verified by negative controls in which the primary antibody was omitted. The whole slide was examined and the total numbers of neutrophils, lymphocytes, and monocytes were counted. The numbers of positive cells were recorded and the percentage positive of each cell type was calculated. As for the MZN, positive slides were confirmed by independent review from another technician and 25% of slides were randomly selected for rereading.

Data Analysis

The diagnostic performance of conventional ZN stain was compared with the MZN stain and ESAT-6 stain against both *M. tuberculosis* culture and clinical gold standard diagnostic criteria (11). The 95% confidence intervals for sensitivity, specificity, and positive and negative predictive value were calculated based on binomial probabilities. The statistical analysis was performed using SPSS (V21.0, IBM, Foster City, CA) and GraphPad PRISM (Graph Pad Software, San Diego, CA). Differences were considered statistically significant when P less than 0.05. Categorical variables were compared by the chi-square test if independent, and McNemar test if paired. Continuous variables were compared by the Mann-Whitney U test.

Results

Three hundred and ten consecutive patients uninfected by HIV with meningitis were screened for entry into the study (Figure 1). Thirty patients were excluded, either because there were incomplete clinical data ($n = 10$), or because they had a TBM diagnostic score of less than six and did not fulfill the minimum entry criteria of “possible” TBM ($n = 20$). ESAT-6 staining

was not performed on 7 patients with probable TBM and 12 with possible TBM because there was too little CSF.

The clinical features at lumbar puncture of the 280 patients enrolled in the study are provided in Table 1. Forty patients were subsequently confirmed to have an alternative diagnosis to TBM. None of the patients were infected with HIV; 172 (61.4%) were male, and the median age of those studied was 34 years (range, 2–87). Forty-one patients (14.7%) were younger than 18 years of age and 35 (12.6%) were older than 60 years. The median duration of symptoms before CSF was taken was 8 days (1–288). All 240 patients with a clinical diagnosis of TBM received antituberculosis drugs; 25 (10.4%) of these patients had more than 1 day of treatment before the CSF was taken and tested. None of the patients with an alternative diagnosis received antituberculosis drugs. In-hospital mortality of all patients enrolled is presented in Table 1.

Performance of Conventional ZN Stain and Culture

The sensitivity of conventional ZN stain and culture was 8 of 240 (3.3%) and 37 of 240 (15.4%), respectively against a clinical gold standard (final diagnostic score > 5) (Table 2). The specificity of CSF ZN stain and culture was 100%.

Patients with a positive conventional ZN stain were significantly more likely to have evidence of lung tuberculosis on chest radiograph (37.5% vs. 10.3%; $P = 0.016$) and have higher numbers of CSF white cells (median, 207 vs. 66 cells per cubic millimeter; $P = 0.009$), a larger number of which were neutrophils (median, 67 vs. 1 cells per cubic millimeter; $P = 0.001$), than those with no AFB seen. AFB were seen in 2 of 25 (8.0%) patients pretreated with more than 1 day of antituberculosis drugs, versus 6 of 215 (2.8%) of untreated patients.

Performance of MZN Stain

The sensitivity of MZN stain was 37 of 37 (100%) against CSF culture and 199 of 240 (82.9%) against clinical diagnostic criteria (Table 2), significantly higher than the respective figures for conventional ZN stain ($P < 0.001$ for both comparisons). Sensitivity was 90.6 and 74.8% in those with probable and possible TBM (Table 3). However, intracellular AFB were also seen in six patients with cryptococcal ($n = 4$) and pyogenic bacterial meningitis ($n = 2$) (see Tables E1 and E2 and Figure E1 in the

Figure 1. The inclusion of participants in the study and the numbers of tests performed in each diagnostic group. ESAT-6 = early secreted antigen target-6; MZN = modified Ziehl-Neelsen; TBM = tuberculous meningitis.

online supplement), yielding a diagnostic specificity of 85.0% (34 of 40).

The median number of fields with AFB seen following MZN was 19 (range, 0–208) of 300 examined (6.3%). There was a strong association between positive conventional ZN stain and culture and the number of MZN AFB positive fields. The number

of positive MZN fields in those with conventional ZN and culture-positive CSF was 120 (range, 82–280) and 45 (range, 8–280), respectively ($P < 0.001$).

Intracellular bacteria were seen in 180 of 205 (87.8%) of positive cases by MZN. The median total number of cells containing AFB was 11 (range, 0–100) in those with

definite TBM, 8 (range, 0–28) with probable TBM, and 3 (range, 0–25) with possible TBM ($P < 0.001$) (Figure 2). A higher proportion of the positive cells were neutrophils (mean, 52.0%) in those with definite TBM compared with those with probable and possible TBM, in whom the positive cells were predominantly monocytes (mean, 50.0 and 80.0%, respectively) (see Figure E3).

In the 37 patients with CSF culture-confirmed TBM, the time to positive culture (a measure of bacterial load in the CSF) was inversely correlated with the numbers (correlation coefficient, -0.43 ; $P = 0.008$) and proportions (correlation coefficient, 0.36 ; $P = 0.042$) of MZN-positive that were neutrophils. There was a trend time-to-culture to be positively correlated with an increased proportion of positive monocytes (correlation coefficient, 0.335 ; $P = 0.061$).

Patients with a positive MZN stain were significantly more likely to have evidence of lung tuberculosis on chest radiograph and have higher numbers of CSF white cells than MZN-negative patients (see Table E3). A positive MZN was not associated with prior antituberculosis treatment: 24 of 25 (96.0%) patients pretreated with more than 1 day of antituberculosis drugs were MZN-positive compared with 175 of 215 (81.4%) untreated patients. Furthermore, 16.2%

Table 1: Comparison of the Baseline Clinical Features and In-patient Outcome of Those with Definite, Probable, and Possible TBM and Those Who Did Not Have TBM

Variables	Definite TBM (n = 37)	Probable TBM (n = 64)	Possible TBM (n = 139)	Not TBM (n = 40)*
Age, yr	29 (3–82)	27 (2–73)	36 (2–87)	41 (3–38)
Male sex	15 (40.5)	44 (68.8)	86 (61.9)	27 (67.5)
Duration of symptoms, d	6 (1–44)	9 (1–205)	7 (1–288)	30 (1–92)
Chest radiograph appearances of active tuberculosis	4 (10.8)	22 (34.4)	5 (3.6)	0
>1 d of antituberculosis treatment before CSF taken	6 (16.2)	18 (28.1)	1 (0.7)	0
MRC disease severity grade				
I	21 (56.8)	46 (71.9)	95 (70.4)	
II	11 (29.7)	12 (18.8)	31 (23.0)	
III	5 (13.5)	6 (9.4)	9 (6.7)	
Glasgow Coma Score, /15	15 (6–15)	15 (8–15)	15 (6–15)	14 (8–15)
CSF total white cell count, /mm ³	140 (7–1,029)	89 (0–3,208)	41 (0–5,420)	51 (0–3,208)
CSF total neutrophils, /mm ³	26 (0–387)	1 (0–2,775)	0 (0–4,688)	4 (0–2,775)
CSF total lymphocytes, /mm ³	75 (3–993)	45 (0–498)	29 (0–995)	26 (0–689)
CSF total monocytes, /mm ³	11 (1–63)	7 (0–385)	4 (0–583)	6 (0–385)
CSF glucose, mmol/L	1.8 (0.4–2.1)	2.5 (0.4–5.4)	3.1 (0.61–6.8)	2.1 (0.1–3.4)
CSF protein, g/dl	1.1 (0.2–2.1)	1.0 (0.3–5.0)	0.6 (0.04–6.5)	1.0 (0.3–2.1)
Death before discharge from hospital	5 (13.5%)	6 (9.4%)	13 (9.4%)	13 (32.5%)

Definition of abbreviations: CSF = cerebrospinal fluid; MRC = Medical Research Council; TBM = tuberculous meningitis.

Data are shown as n (%) (Death before discharge from hospital) or mean (95% confidence interval) (all other rows).

*A total of 36 had cryptococcal meningitis (India ink positive CSF) and 4 had CSF culture-confirmed pyogenic bacterial meningitis.

Table 2: Summary of Performance of Conventional ZN Stain, Modified ZN Stain, and ESAT-6 Immunocytochemical Stain in Those with Definite TBM, Definite and Probable TBM, and Definite, Probable, and Possible TBM

Gold Standard	Diagnostic Performance Measure	Conventional ZN Stain	Modified ZN Stain	ESAT-6 Immunocytochemical Stain
Definite TBM (CSF <i>Mycobacterium tuberculosis</i> culture positive)	Sensitivity, %	21.1 (10.4–38.7)	100 (88.2–100)	81.1 (64.2–91.4)
	Specificity, %	100 (89.1–100)	85 (69.4–93.7)	90.0 (75.4–96.7)
	Positive predictive value	1.00 (0.60–1.00)	0.86 (0.71–0.94)	0.88 (0.72–0.96)
	Negative predictive value	0.58 (0.45–0.70)	1.00 (0.87–1.00)	0.84 (0.69–0.93)
Definite and probable TBM by clinical criteria	Sensitivity, %	7.9 (3.5–15.0)	94.1 (87.5–97.8)	80.9 (71.4–88.2)
	Specificity, %	100 (91.1–100)	85.0 (70.2–94.3)	90.0 (76.3–97.2)
	Positive predictive value	1.00 (0.63–1.00)	0.94 (0.88–0.98)	0.95 (0.88–0.99)
	Negative predictive value	0.30 (0.22–0.39)	0.85 (0.70–0.94)	0.67 (0.53–0.79)
Definite, probable, or possible TBM by clinical criteria	Sensitivity, %	3.3 (1.6–6.7)	82.9 (77.4–87.3)	75.1 (68.8–80.6)
	Specificity, %	100 (89.1–100)	85.0 (69.4–93.8)	90.0 (75.4–96.7)
	Positive predictive value	1.00 (0.60–1.00)	0.97 (0.93–0.99)	0.98 (0.94–0.99)
	Negative predictive value	0.15 (0.11–0.20)	0.45 (0.34–0.57)	0.40 (0.30–0.50)

Definition of abbreviations: CSF = cerebrospinal fluid; ESAT = early secreted antigen target; TBM = tuberculous meningitis; ZN = Ziehl-Neelsen. Data are shown as mean (95% confidence interval).

(6 of 37) of culture-confirmed cases had more than 1 day of antituberculosis treatment compared with 10.7% (18 of 168) who were MZN-positive and culture/ZN stain-negative ($P = 0.35$). There was no significant relationship between a positive MZN stain and disease severity or Glasgow Coma Score at baseline.

Performance of ESAT-6 Immunocytochemical Stain

The sensitivity of CSF ESAT-6 white cell stain was 81.1% against CSF culture and 75.1% against a clinical diagnostic gold standard (Table 2). Sensitivity varied from 81.1% in those with definite TBM to 80.7 with probable TBM, and 70.9% with possible TBM (Table 3). Six patients had a positive ESAT-6 stain and an alternative diagnosis (specificity, 85.0%), although two

were also positive by MZN, suggesting possible mixed infections in these patients. Treatment details for these six patients are provided in Table E1.

The median total number of ESAT-6-staining cells was three per cubic millimeter (range, 0–1,268), an average of 11.5% of all CSF white cells. More CSF white cells were positive in those with definite TBM (median, 11; range, 0–210) than those with probable TBM (median, 1; range, 0–1,268) or possible TBM (median, 2; range, 0–361) (Figure 2). Monocytes stained most frequently with ESAT-6, especially in those with probable or possible TBM.

As for a positive MZN, patients with a positive ESAT-6 stain were significantly more likely to have evidence of lung tuberculosis on chest radiograph and have

higher numbers of CSF white cells than MZN-negative patients (see Table E3). The time-to-positive culture of *M. tuberculosis* from CSF was inversely correlated with the total number of ESAT-6-staining cells (correlation coefficient, -0.45 ; $P = 0.005$). Prior antituberculosis treatment did not influence the results of the test: 17 of 25 (68.0%) were ESAT-6-positive despite more than 1 day of antituberculosis treatment, compared with 149 of 215 (69.3%) untreated patients.

Combined Performance of MZN and ESAT-6 Stains

In patients in whom both tests were performed ($n = 221$), and a positive result from either accepted, the combined diagnostic sensitivity was 90.0% (95% confidence interval, 84.6–93.1%), positive

Table 3: Comparison of Diagnostic Performance of Conventional ZN Stain, MZN Stain, and ESAT-6 Stain Alone and in Combination across the Diagnostic Groups

Test	Definite TBM (n = 37)	Probable TBM (n = 64)	Possible TBM (n = 139)	Not TBM (n = 40)
Conventional ZN stain positive	8 (21.6%)	0	0	0
MZN positive	37 (100%)	58 (90.6%)	104 (74.8%)	6 (15.0%)
ESAT-6 stain positive	30 (81.1%)	46/57 (80.7%)	90/127 (70.9%)	4 (10.0%)
MZN and ESAT-6 stain positive	30 (81.1%)	43/57 (75.4%)	75/127 (59.1%)	2 (5.0%)
MZN positive and ESAT-6 stain negative	7 (18.9%)	8/57 (14.0%)	17/127 (13.4%)	4 (10.0%)
MZN negative and ESAT-6 stain positive	0	3/57 (5.3%)	15/127 (11.8%)	2 (5.0%)
MZN positive or ESAT-6 positive (both tests performed)	37 (100%)	54/57 (94.7%)	107/127 (84.3)	8 (20%)
MZN and ESAT-6 stain negative	0	3/57 (5.3%)	20/127 (15.7%)	32 (80.0%)

Definition of abbreviations: CSF = cerebrospinal fluid; ESAT = early secreted antigen target; MZN = modified Ziehl-Neelsen; TBM = tuberculous meningitis; ZN = Ziehl-Neelsen.

Figure 2. The number of positive fields, intracellular bacteria, and early secreted antigen target (ESAT)-6–stained cells seen in cerebrospinal fluid following (a) conventional Ziehl-Neelsen stain, (b) modified Ziehl-Neelsen stain (MZN), and (c) the ESAT-6 stain.

predictive value 0.96 (95% confidence interval, 92.2–98.1), and negative predictive value 0.58 (95% confidence interval, 0.44–0.71).

Discussion

The rapid diagnosis of TBM has challenged physicians ever since the advent of antituberculosis drugs in the late 1940s. Many different laboratory assays have been studied to improve on conventional CSF ZN stain and microscopy, including nucleic acid amplification (13); the detection of specific mycobacterial antibodies and antigens (14); and the concentration of other CSF molecules, such as adenosine deaminase (15). None has been proved to combine the requisite attributes of high sensitivity and specificity, with speed and ease of use.

We describe a simple modification to the CSF ZN stain that seems to transform its diagnostic performance. Using cytopsin to prepare a monolayer of CSF white cells on a slide, and permeabilizing the cells with TritonX-100 before and during the ZN stain, AFB were stained and visualized by light microscopy in 82.9% of patients with TBM. Intracellular bacteria were seen in

87.8% of positive specimens. Against a clinical diagnostic gold standard (11), the sensitivity of conventional CSF ZN stain was 3.3% (95% confidence interval, 1.6–6.7%) compared with 82.9% (95% confidence interval, 77.4–87.3%) for MZN (Table 2). These findings confirm the results of an earlier pilot study that found AFB were seen in the CSF by MZN in all 48 specimens taken from 29 patients with TBM.

In parallel, we also investigated the performance of a new diagnostic assay, ESAT-6 immunocytochemical staining of CSF white cells, and found that it was marginally less sensitive than MZN (75.1%; 95% confidence interval, 68.8–80.6%). However, if the results of the two assays were combined, and either positive result accepted, the diagnostic sensitivity rose to 90.0% (95% confidence interval, 84.6–93.1%) against a clinical diagnostic gold standard.

There were, however, a relatively high number of patients with apparent false-positive MZN (6 of 40) and ESAT-6 (4 of 40) stains, which is a potentially important limitation of these new tests that needs to be explained by future studies. We did not use a negative control for either of these stains, because of the difficulty of obtaining

appropriate known-negative fresh CSF; therefore, laboratory artifact and nonspecific staining cannot be excluded. All these patients had a microbiologically confirmed alternative diagnosis to TBM. Therefore, the presence of intracellular AFB either represented true mixed infection (which is considered extremely rare), or was artifact. In general, there were small numbers of bacteria or ESAT-6–stained cells seen in these patients (Figures 2; see Figures E2 and E4) but there were insufficient data to analyze whether a useful threshold might exist to better define true- or false-positive slides. Future studies should explore this possibility. Furthermore, we do not believe the AFB seen is likely to represent latent bacteria, uninvolved in the disease. In contrast, it is plausible ESAT-6–positive monocytes from latently infected individuals might migrate into the CSF in those with meningitis of another cause. These possibilities need to be explored by evaluating the assays in a larger cohort of patients with non-TBM.

Our findings suggest that these two new diagnostic approaches could revolutionize the diagnosis of TBM, although they invite some important questions. In particular, why were bacteria seen in CSF following MZN in 82.9% of patients, but *M. tuberculosis* was only cultured from 18.6% of these cases. We speculate that intracellular bacteria may have been seen, but not cultured, because they were dead. Antituberculosis chemotherapy started before the lumbar puncture may have been responsible for their death, although this seems unlikely because a higher proportion of ZN/culture-positive patients received prior antituberculosis chemotherapy than those MZN-positive alone. Alternatively, the bacteria may have been killed by the phagocytic cells. In patients with culture-confirmed TBM, AFB was predominantly seen within neutrophils, whereas in those with culture-negative disease the bacteria were mostly found within monocytes (see Figure E2). Recent data suggest that human neutrophils are relatively poor killers of *M. tuberculosis* compared with monocytes and macrophages (16), which may explain why AFB contained predominantly within monocytes failed to grow. There is a complex and poorly understood relationship between the numbers and types of leukocytes in the CSF, the bacterial load, and

clinical outcome from TBM. Other studies have shown that high numbers of neutrophils in the CSF are associated with more bacteria seen, yet, paradoxically, better clinical outcomes (17). The MZN stain therefore provides an opportunity to better examine these relationships, and to identify the important immunologic determinants of outcome in the quest for more effective adjunctive therapy.

In summary, we have developed and tested two new diagnostic tests for TBM, both of which show great promise. In particular, simple modifications to the CSF ZN stain, which could be widely and rapidly adopted by laboratories worldwide, might dramatically improve the diagnosis of this lethal infection. Future studies of these tests must include larger numbers of patients without TBM to provide more precise estimates of specificity, compare their

performance against commercial nucleic acid amplification tests (e.g., GeneXpert MTB/RIF), and investigate whether the auramine stain can be similarly adapted. ■

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