JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2004, p. 996–1002 Vol. 42, No. 3
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.3.996–1002.2004

Comparison of Conventional Bacteriology with Nucleic Acid Amplification (Amplified Mycobacterium Direct Test) for Diagnosis of Tuberculous Meningitis before and after Inception of Antituberculosis Chemotherapy

Guy E. Thwaites,[1,2]* Maxine Caws,[1,2] Tran Thi Hong Chau,[3] Nguyen Thi Dung,[3] James I. Campbell,[1,2] Nguyen Hoan Phu,[3] Tran Tinh Hien,[3] Nicholas J. White,[1,2] and Jeremy J. Farrar[1,2]

Oxford University Clinical Research Unit, Hospital for Tropical Diseases,[1] and Hospital for Tropical Diseases,[3] Ho Chi Minh City, Vietnam, and Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford OX3 7LJ, United Kingdom[2]

Received 22 October 2003/Returned for modification 4 December 2003/Accepted 16 December 2003

The role of nucleic acid amplification techniques in the rapid diagnosis of tuberculous meningitis remains uncertain. We compared the performance of Ziehl-Neelsen (ZN) staining, the Gen-Probe amplified *Mycobacterium tuberculosis* direct test (MTD), and culture with 341 cerebrospinal fluid specimens from 152 adults (73 with and 79 without tuberculous meningitis) before and after inception of antituberculosis chemotherapy. The sensitivity, specificity, and positive and negative predictive values of ZN staining before treatment were 34/66 (52%), 79/79 (100%), 34/34 (100%), and 79/111 (71%), compared with 25/66 (38%), 78/79 (99%), 25/26 (96%), and 79/120 (66%) for MTD. The sensitivity of combined ZN staining and MTD (either positive) was 45/66 (68%). The sensitivity of staining and culture fell more rapidly than that of MTD after the start of treatment: after 5 to 15 days of treatment, MTD was more sensitive than ZN staining (12/43 [28%] versus 2/43 [2%]; $P = 0.013$). Slower bacterial clearance was observed if *M. tuberculosis* was resistant to isoniazid and/or streptomycin: resistant organisms were more likely to be cultured from cerebrospinal fluid after 2 to 5 days of treatment than fully sensitive organisms ($P < 0.001$). The sensitivities of ZN staining, MTD, and the two tests combined were improved by repeated sampling to 38/59 (64%), 35/59 (59%), and 49/59 (83%), respectively. In conclusion, ZN staining of the cerebrospinal fluid is at least as good as MTD for the rapid diagnosis of tuberculosis and is much faster and less expensive. However, the combination of these methods on serial samples detects more cases. Alternative tests are still urgently required.

There is an urgent need to improve rapid diagnostic methods for tuberculous meningitis (TBM). Death from TBM is strongly associated with delays in diagnosis and treatment. Despite modern antituberculosis chemotherapy (ATC), 30% of patients still die and many of the remainder have a significant neurological deficit (10). The search for acid-fast bacilli (AFB) in the cerebrospinal fluid (CSF) remains the most widely available rapid diagnostic test, but the reported sensitivity of this approach varies enormously (20). Excellent results can be achieved, but this requires large volumes of CSF and careful microscopy (12, 19, 21). Alternative rapid methods that overcome the limitations of conventional bacteriology may improve patient care and survival.

Early reports suggested that the amplification of nucleic acid specific to *Mycobacterium tuberculosis* from the CSF of patients with TBM might improve upon conventional bacteriology (11, 18). A recent systematic review and meta-analysis of the accuracy of nucleic acid amplification tests for the diagnosis of TBM concluded that these tests were specific but insensitive (the sensitivity of commercial assays was 56% [95% confidence interval {CI}, 46 to 66%]) (16). There are a number of reasons why these methods have failed to meet initial expectations. First, a wide variety of methods have been reported, many of which were developed in-house without adequate standardization, and conclusions regarding their general applicability are difficult. Second, few studies have been able to compare performance against a bacteriological "gold standard," instead choosing a variety of clinical diagnostic criteria that have never been assessed prospectively. Last, TBM is rare in most settings capable of applying the required technology; consequently, most published studies are small, reporting few patients with TBM, and lack the statistical power to demonstrate unequivocal differences in performance.

The Food and Drug Administration (FDA) of the United States has licensed two assays for the direct detection of *M. tuberculosis* nucleic acid in smear-positive respiratory samples: the amplified *M. tuberculosis* direct test (MTD) (Gen-Probe, Inc., San Diego, Calif.) and the AMPLICOR *M. tuberculosis* test (Roche Diagnostics, Inc., Indianapolis, Ind.). Gen-Probe modified the MTD to enhance sensitivity and decrease the time to obtain results, and in 1999 the FDA extended the license of this product to include all respiratory samples, regardless of smear result. However, neither of these kits is licensed for use on nonrespiratory samples, and there are limited data on their performance with CSF. Studies using the

* Corresponding author. Mailing address: The University of Oxford Clinical Research Unit, The Hospital for Tropical Diseases, 190 Ben Ham Tu, Quan 5, Ho Chi Minh City, Vietnam. Phone: 84 88 923 7954. Fax: 84 88 923 8904. E-mail: gthwaites@hcm.vnn.vn.

TABLE 1. Published studies using the Gen-Probe MTD test with CSF for diagnosis of TBM

Authors (reference)	Setting	MTD kit used[a]	No.					% MTD sensitivity (95% CI)	% MTD specificity (95% CI)
			CSF specimens	TBM CSF specimens	AFB in CSF	Positive cultures	MTD positive		
Chedore and Jamieson (5)	Canadian reference lab	II	311	16	7	16	15	93[b] (70–100)	99 (98–100)
Baker et al. (1)	U.S. inner city hospital	II	29	9	0	5	5	56 (21–86)	100 (86–100)
Lang et al. (13)	Dominican Republic[c]	I	84	19	0	5	4	33 (17–55)	100 (94–100)
Gamboa et al. (8)	Spanish hospital	I	22	8	0	8	5	63 (26–90)	100 (77–100)
Gamboa et al. (9)	Spanish hospital	I	17	8	0	8	5	63 (26–90)	100 (66–100)
Ehlers et al. (7)	German hospital	I	51	6	1	Not given	4	67 (24–94)	98 (87–100)
Pfyffer et al. (17)	Swiss hospital	I	54	6	1	5	6	100 (54–100)	96 (85–99)
Total			568	72	9	47	44	61 (49–72)	99 (98–100)

[a] I, original MTD kit; II, enhanced MTD.
[b] No data on clinical diagnosis were given.
[c] The assay was done in the United States with frozen CSF.

Roche AMPLICOR assay suggest that it does not improve upon microscopy for AFB in the CSF (2, 3, 4).

The MTD amplifies target *M. tuberculosis* rRNA by transcription at a constant temperature and detects the amplicon by probing with chemiluminescence-labeled complementary DNA. A luminometer detects stable RNA-DNA hybrids. There have been seven published studies that report the use of MTD for the diagnosis of TBM (Table 1). These data suggest that it may be more sensitive than other nucleic acid amplification tests (pooled sensitivity, 61%; 95% CI, 49 to 72%), but only 72 TBM CSF specimen results have been published (Table 1). Larger studies are therefore required before the MTD and other nucleic acid amplification assays can be recommended for routine diagnostic use.

The effect of ATC on the performance of conventional and molecular diagnostic methods is also uncertain. There is evidence to suggest that repeated sampling after the start of ATC improves the diagnostic yield of CSF Ziehl-Neelsen (ZN) staining and culture (12). There are also data that suggest that *M. tuberculosis* DNA can be detected in the CSF for at least 4 weeks after the start of treatment (6). Also, organisms resistant to first-line drugs may be more readily detected by all methods after treatment, but this issue remains unexamined in patients with TBM.

The aims of our study were to assess the performance of the MTD against conventional bacteriology before and after the start of ATC and to investigate the impact of drug-resistant *M. tuberculosis* on the sensitivity of these methods after the start of treatment.

MATERIALS AND METHODS

Setting. The adults in this study were consecutively admitted to the Clinical Research Unit at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, between October 2000 and October 2002. The Hospital for Tropical Diseases is a 500-bed infectious diseases hospital serving the local community and acts as the tertiary referral center for infectious diseases for southern Vietnam. The hospital treats approximately 30,000 inpatients, and 52,000 outpatients each year. The Clinical Research Unit is a 15-bed ward for adults with central nervous system infection, severe malaria, sepsis, and acute renal failure. The hospital Scientific and Ethical Committee approved the study.

Patients. All adults entering the ward with possible TBM were eligible to enter the study. The clinical and laboratory features and final diagnosis of each patient admitted were recorded prospectively in individual study notes. The diagnosis of TBM was confirmed if AFB were seen or *M. tuberculosis* was cultured from the CSF; the diagnosis was considered probable if AFB or *M. tuberculosis* was found at another site or if there was evidence of active extraneural tuberculosis; and TBM was considered possible if the history was longer than 5 days and the CSF abnormalities included a raised white cell count, lymphocyte predominance, and low CSF/blood glucose ratio (<0.5). The diagnosis of TBM was excluded if another pathogen was seen or cultured from the CSF or the patient recovered fully without ATC. All patients with TBM were tested for antibodies to human immunodeficiency virus (HIV).

Treatment consisted of daily intramuscular streptomycin (20 mg/kg; maximum, 1 g) and oral isoniazid (5 mg/kg), rifampin (10 mg/kg), and pyrazinamide (30 mg/kg) for 3 months, followed by three drugs (isoniazid, rifampin, and pyrazinamide) for 6 months. Outcome (death or survival) was assessed after 9 months of treatment.

Routine investigations. It is routine clinical practice in the Clinical Research Unit for all patients with TBM to have a lumbar puncture at diagnosis (day 0) and at days 3, 7, 30, 60, and 270 of treatment unless this is clinically contraindicated. CSF cell counts and biochemistry were performed by standard methods for each sample, as were Gram and India ink staining with culture for fungi and pyogenic bacteria.

CSF ZN staining and culture. The attending physicians were encouraged to submit 5 to 10 ml of CSF from any adult admitted with possible TBM. The volume of each CSF sample submitted was recorded, the sample was centrifuged immediately at 3,000 × *g* for 15 min, and the resuspended sediment (approximately 600 μl) was divided equally into three parts for ZN staining, culture, and MTD. CSF staining and culture in liquid mycobacterial growth indicator tubes (Becton Dickinson) and on Lowenstein-Jenson medium were performed immediately according to methods described previously (21). Aliquots of sediment reserved for MTD were frozen at −70°C prior to testing.

Adaptation of MTD for CSF. This study used the enhanced amplified MTD, and all tests were performed blind to the ZN staining and culture results. Pfyffer et al. reported low sensitivity when using the MTD with untreated spiked CSF and demonstrated improved sensitivity following pretreatment with sodium dodecyl sulfate (SDS)-NaOH (5×10^5 cells/ml detected with untreated CSF and 2×10^2 cells/ml detected with treated CSF) (17). These experiments were repeated in our laboratory before MTD testing was started. CSF from a patient with culture-confirmed cryptococcal meningitis was divided into 450-μl aliquots and spiked with 50 μl of a 1/10 dilution series of a clinical *M. tuberculosis* isolate (in phosphate-buffered saline) in triplicate. Each dilution series was then subjected to one of three protocols: centrifugation and resuspension in 450 μl of phosphate buffer according to the package insert protocol for sputum, centrifugation and resuspension in 100 μl of phosphate buffer (in order to increase the rRNA concentration in the lysed sample), and pretreatment according to the method of Pfyffer et al. (17). Briefly, distilled H_2O was added to the sample to make 500 μl, followed by addition of 500 μl of 3.16% SDS–1% NaOH. This mixture was vortexed, incubated for 40 min, and then neutralized with 500 μl of 1.43% H_3PO_4 and washed with 500 μl of distilled H_2O. The deposit was then resuspended in 450 μl of phosphate buffer and subjected to MTD according to the manufacturer's protocol. The effect of 1-, 2-, 3-, and 4-h amplification incubation times on sensitivity was also tested with spiked CSF. Positive and negative cell

TABLE 2. Admission clinical data for 59 adults with TBM included in the study before the start of ATC

Variable	No. (%) or median (range)
Male sex	30 (50.8)
Age (yr)	33 (15–69)
HIV infection	5 (8.5)
Medical Research Council disease severity grade[c]	
I	17 (28.8)
II	29 (49.2)
III	13 (22.0)
Duration of symptoms before treatment (days)	15 (5–35)
Vol of CSF (ml)	4.0 (1–12)
CSF opening pressure (cm H_2O)	23 (6–40)
CSF total white cells (10^3/ml)	461 (1–1,750)
% Neutrophils	27 (0–90)
% Lymphocytes	73 (10–100)
CSF protein (mg/dl)	169 (66–4700)
CSF lactate (mmol/liter)	5.8 (1.1–16.4)
CSF chloride (mmol/liter)	104 (84–129)
CSFblood glucose ratio	0.25 (0.05–0.67)
9-mo survival	45 (76.3)

[a] See reference 14.

controls were included with each run according to the manufacturer's recommendations, as were two additional phosphate buffer negative controls. The results were interpreted according to Gen-Probe specifications: a value of >500,000 relative light units was considered positive, <30,000 was considered negative, and 30,000 to 499,999 was deemed equivocal and the sample was retested.

Statistical analysis. The paired proportions of positive tests before and after treatment were compared by use of McNemar's test. Unpaired proportions were compared by use of the chi-square test. The analysis was performed with Stata, version 6.0 (StataCorp LP, College Station, Tex.).

RESULTS

Adaptation of the MTD to CSF. The limit of detection for untreated spiked CSF was 3×10^3 cells/ml. Resuspension of CSF in a smaller volume of lysis buffer decreased the sensitivity to $>3 \times 10^4$ cells/ml, and pretreatment according to the method of Pfyffer et al. improved detection to 30 cells/ml. Experimentation with the enzyme incubation period suggested that 3 h gave the best sensitivity. Consequently, the protocol for testing all of the clinical specimens was adapted to include pretreatment and 3 h of enzyme incubation.

TABLE 3. Admission clinical data for 79 adults without TBM

Variable	No. (%) or median (range)
Male sex	48 (60.7)
Age (yr)	37 (15–75)
HIV infection	8 (10)
Duration of symptoms before treatment (days)	10 (5–27)
Vol of CSF (ml)	3.5 (0.5–10)
CSF opening pressure (cm of H_2O)	21 (8–40)
CSF total white cells (10^3/ml)	780 (5–3050)
% Neutrophils	35 (0–95)
% Lymphocytes	62 (10–100)
CSF protein (mg/dl)	125 (66–230)
CSF lactate (mmol/liter)	3.8 (1.6–12.0)
CSF chloride (mmol/liter)	114 (93–125)
CSF/blood glucose ratio	0.35 (0.10–0.55)

Specimens, clinical data and controls. A ZN staining, a culture for *M. tuberculosis*, and the MTD were performed on 341 CSF samples from 152 adults admitted consecutively to the ward. Of these, 262 specimens came from 73 adults treated for TBM, and serial samples taken before and after ATC were available from 59 patients. CSF specimens from 14 adults who started chemotherapy before the start of the study were also included. The admission clinical and laboratory findings are shown in Table 2. A bacteriological diagnosis was confirmed for 57 of 73 adults (78%), 11 of 73 (15%) had probable TBM, and 4 of 73 (7%) had possible TBM. The diagnosis of TBM was excluded in 79 adults, and 79 admission CSF specimens from these patients served as negative controls. These adults were diagnosed as having pyogenic bacterial meningitis (n = 20), pyogenic brain abscess (n = 1), viral meningoencephalitis (n = 22), mumps meningitis (n = 1), eosinophilic meningitis (n = 12), cryptococcal meningitis (n = 20), and subarachnoid hemorrhage (n = 2). The admission clinical and laboratory findings for this group are shown in Table 3.

Diagnostic performance before antituberculosis treatment. The sensitivities of CSF ZN staining (52%) and MTD (38%) were not significantly different before the start of treatment (P = 0.150). The sensitivity, specificity, and positive and negative predictive values for each of the tests are shown in Table 4. ZN staining was 52% sensitive (95% CI, 39 to 64%) and 100% specific against a clinical diagnostic gold standard and was 57% sensitive (95% CI, 41 to 72%) and 90% specific against a culture gold standard. AFB were seen but not cultured from eight specimens, all of which were from adults with evidence of extraneural tuberculosis who responded appropriately to antituberculosis drugs. The MTD was 38% sensitive (95% CI, 26 to 51%) and 99% specific against a clinical gold standard and was 50% sensitive (95% CI, 34 to 66%) and 95% specific against culture gold standard.

Table 5 presents the numbers of discrepant and concordant positive results with ZN staining and MTD and their combined sensitivity before and after inception of treatment. Before treatment, the MTD was negative in 20 of 34 ZN-positive specimens (59%), but the ZN staining was negative in 11 of 25 specimens (44%) that were positive by MTD. The combined pretreatment sensitivity of ZN staining and MTD (ZN positive and/or MTD positive) was 45 of 66 (68%), but only 14 of 45 specimens (31%) were positive by both tests.

There was one false-positive MTD with the CSF of an adult believed to have viral meningoencephalitis. The patient was admitted with a 5-day history of fever and headache; the CSF contained 150×10^3 cells/ml, mostly lymphocytes, and the CSF glucose was normal. The patient made a complete recovery in the hospital without antituberculosis treatment and was contacted and found to be well 18 months after admission, excluding a diagnosis of TBM.

Diagnostic performance after inception of ATC. The sensitivity of ZN staining fell faster with treatment than did that of MTD (Table 5). The sensitivity of MTD did not fall between days 2 and 5 of therapy, and MTD was nearly six times more sensitive than ZN staining after 5 to 15 days of treatment (28 versus 5%; 95% CI of difference, 41 to 5%; P = 0.013). Agreement between ZN stainings and positive MTD results wors-

TABLE 4. Sensitivity, specificity, and positive and negative predictive values of CSF ZN staining, MTD, and culture before the start of ATC

Gold standard	Test	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]	Positive predictive value (%) [95% CI]	Negative predictive value (%) [95% CI]
Clinical diagnostic[a]	ZN staining	34/66 (52) [39–64]	79/79 (100) [95–100]	34/34 (100) [90–100]	79/111 (71) [62–79]
	MTD	25/66 (38) [26–51]	78/79 (99) [95–100]	25/26 (96) [80–100]	79/120 (66) [57–74]
	Culture	38/66 (58) [45–70]	79/79 (100) [95–100]	38/38 (100) [91–100]	79/107 (74) [64–81]
Culture[b]	ZN staining	24/42 (57) [41–72]	71/79 (90) [81–95]	24/34 (71) [52–85]	79/97 (81) [72–88]
	MTD	21/42 (50) [34–66]	75/79 (95) [88–98]	21/25 (84) [64–95]	79/97 (81) [72–88]
	Culture	38/42 (90) [77–97]	79/79 (100) [95–100]	38/38 (100) [91–100]	79/83 (95) [88–99]

[a] Definite, probable, and possible TBM.
[b] *M. tuberculosis* isolated from the CSF at any time before or after start of ATC.

ened after the start of treatment: both tests were positive in 5 of 17 specimens (29%) positive by either test taken 2 to 5 days after treatment and in 0 of 16 positive specimens after 5 days.

Table 6 shows the number of new positive tests on repeated CSF samples from 59 patients before and after the start of treatment for TBM. Testing of a repeat CSF specimen collected at day 2 to 5 improved the overall sensitivity of ZN staining by 3/33 (9%) and that of MTD by 10/24 (42%). Repeated sampling resulted in a cumulative sensitivity of 38/59 (64%; 95% CI, 51 to 76%) for ZN staining, 35/59 (59%; 95% CI, 46 to 72%) for MTD, and 42/59 (71%; 95% CI, 71 to 92%) for culture against a clinical gold standard. The cumulative sensitivity of ZN staining and MTD combined was 49/59 (83%; 95% CI, 71 to 92%).

A positive ZN staining or MTD during the first 80 days of treatment was associated with examination of larger volumes of CSF. The median volume taken from those with either test positive (78 of 222) was 4.0 ml (range, 1 to 12 ml), compared with 3.0 ml (range, 2 to 8 ml) from those with both tests negative (144 of 222) ($P = 0.019$).

Impact of drug resistance on diagnostic performance. Table 7 presents the effect of resistance on the performance of ZN staining, MTD, and culture before and after the start of treatment. Ten isolates were resistant to streptomycin alone, one was resistant to isoniazid alone, and six were resistant to both isoniazid and streptomycin, and one patient had multidrug-resistant *M. tuberculosis* (resistant to rifampin, isoniazid, and streptomycin) and died before further samples could be taken. The 9-month survival for those with sensitive *M. tuberculosis* (22 of 28; 79%) was not significantly different from the 9-month survival for those with a drug-resistant isolate (14 of 18; 78%).

Before the start of treatment there were no significant differences between the sensitivities of ZN staining, MTD, and culture for patients with sensitive or resistant *M. tuberculosis* (Table 7). After 2 to 5 days of treatment, 2 of 11 patients (18%) with sensitive *M. tuberculosis* were positive by ZN staining, compared with 4 of 10 (40%) with resistant isolates ($P = 0.269$). Drug-resistant *M. tuberculosis* was cultured from all (10 of 10) specimens after 2 to 5 days of ATC, compared with 18% (2 of 11) for fully sensitive isolates ($P < 0.001$). The MTD was positive for 5 of 12 specimens (42%) at 5 to 15 days after treatment if the *M. tuberculosis* was drug resistant and for 4 of 14 (28%) if the isolate was sensitive ($P = 0.484$).

DISCUSSION

The diagnosis of TBM is difficult because the presenting clinical features are nonspecific and the CSF taken may contain so few bacilli that they are neither seen nor cultured, but early diagnosis and treatment of TBM save lives and probably reduce neurological deficits, so they are of paramount importance. A sensitive and specific rapid diagnostic test is required urgently.

The detection of *M. tuberculosis* nucleic acid in the CSF has been possible for more than 10 years, but its role in the rapid diagnosis of TBM remains poorly defined. The aim of this study was to compare conventional bacteriology with the de-

TABLE 5. Number of positive tests before and after start of ATC, with concordant and discordant results of ZN staining and MTD over the first 40 days of treatment

Time of treatment	No. of patients/no. of specimens	No. of specimens positive by[a]:			No. of specimens that were[b]:			
		ZN staining	MTD	Culture	MTD+ and ZN−	MTD− and ZN+	MTD+ and ZN+	MTD+ and/or ZN+
Pretreatment	59/66	34/66 (52)	25/66 (38)	38/66 (58)	11/66 (17)	20/66 (30)	14/66 (21)	45/66 (68)
Days 2 to 5	34/34	8/34 (24)	14/34 (41)	12/34 (35)	9/34 (26)	3/34 (9)	5/34 (15)	17/34 (50)
Days 6 to 15	43/43	2/43 (5)	12/43 (28)	6/43 (14)	12/43 (28)	2/43 (5)	0/43	14/43 (33)
Days 16 to 40	48/48	0/48	2/48 (4)	0/48	2/48 (4)	0/48	0/48	2/48 (4)
Days 41 to 80	35/35	0/35	0/35	1/35 (3)				
Days 260 to 280	36/36	0/36	0/36	0/36				

[a] Each value is the number of positive specimens/total number of specimens. Values in parentheses are percentages. Where ZN staining and MTD results differed, the 95% confidence intervals were as follows (*P* values are in parentheses): for the pretreatment period, −4 to 31% (0.150); for days 2 to 5, −40 to 4% (0.146); and for days 6 to 15, −41 to −5% (0.013).
[b] Each value is the specimens with the indicated results/total number of specimens. Values in parentheses are percentages. MTD+, MTD positive; MTD−, MTD negative; ZN−, ZN staining negative; and ZN+, ZN staining positive.

TABLE 6. New positive tests before and after start of ATC for 59 adults with serial samples

Test	No. positive						Total positive/total no. of specimens (% positive) [95% CI]
	Pretreatment	Days 2 to 5	Days 5 to 15	Days 16 to 40	Days 41 to 80	Days 260 to 280	
ZN staining	34	3	1	0	0	0	38/59 (64) [51–76]
MTD	24	10	1	0	0	0	35/59 (59) [46–72]
ZN staining and/or MTD	45	4	0	0	0	0	49/59 (83) [71–92]
Culture	38	3	1	0	0	0	42/59 (71) [58–82]

tection of *M. tuberculosis* rRNA in the CSF by using the Gen-Probe MTD. The strength of the study is the large number of serial CSF samples taken before and after treatment from patients with a confirmed bacteriological diagnosis of TBM. The MTD was chosen because it is a universally available commercial test and is recognized by the FDA to perform with sufficient sensitivity and specificity to be granted a license for use with smear-negative sputum. There are no commercial tests currently with an FDA license for use with extrapulmonary samples.

Our data show that before the start of treatment the sensitivity of ZN staining is greater than that of MTD (52 versus 38%) against a clinical diagnostic gold standard, although the difference is not significant ($P = 0.150$) (Table 4). It has long been recognized that the sensitivity of CSF ZN staining can exceed 50%, although modern laboratories and many textbooks rarely report this level of performance. We have previously shown that the sensitivity of conventional bacteriology is dependent on the volume of CSF examined and the duration of microscopy (21). The factors that govern the performance of MTD are uncertain. The combined pretreatment sensitivity of ZN staining and MTD was 45/66 (68%), with only 14 of 45 specimens (31%) positive by both tests (Table 5). Analysis of the discordant results between the tests reveals that MTD was negative in 20 of 34 ZN-positive specimens (59%) and that ZN staining was negative in 11 of 25 MTD-positive specimens (44%) (Table 5). It is difficult to understand why rRNA cannot be detected in CSF in which AFB have been seen. Pfyffer et al. found that high concentrations of organisms ($>5 \times 10^5$cells/ml) were required in spiked CSF to obtain a positive MTD result, and they suggested that interference from unknown compounds in the CSF caused assay inhibition (17). We have repeated these experiments and report similar results. Pfyffer et al. also found that increasing the volume of sample used, pretreatment with a denaturing agent such as SDS-NaOH, and increasing the amplification time from 2 to 3 h could improve MTD sensitivity with CSF. Our experimentation with the assay procedures concurs with these findings and suggests that inadequate rRNA extraction from small numbers of organisms in the CSF may be a factor in producing false-negative MTD tests. The existence and impact of factors inhibitory to nucleic acid amplification in CSF remain unclear, but if present these factors are likely to be substantially reduced by the detergent and washing actions of the pretreatment protocol, which may partly explain the improvement in sensitivity produced by these steps. Freezing the CSF for later testing may also reduce sensitivity, although the high numbers of ZN stain-negative, MTD-positive specimens (11 of 25 [44%]) before treatment suggests an alternative explanation. There is a tendency for *M. tuberculosis* bacilli to stick together in clinical samples, and in our experience it is uncommon to see single bacilli in the CSF. This characteristic suggests that bacilli are not evenly distributed through the sample and increases the chance of the divided deposit (for staining, MTD, and culture) containing different concentrations of bacilli. These sampling effects become particularly important when the CSF contains few bacilli and both tests are performing close to their limits of detection. This may explain the greater combined sensitivity of ZN staining and MTD: the larger the volume of sample tested by either test, the larger the chance of detecting *M. tuberculosis*. To support this hypothesis, we have shown that a positive ZN staining or MTD is associated with larger volumes of examined CSF ($P = 0.019$).

Following the start of antituberculosis treatment, the sensitivities of ZN staining and culture fell rapidly (Table 5). The sustained sensitivity of MTD presumably results from dead, nonculturable bacilli. The value of repeated lumbar punctures after the start of treatment is still debated, but our data suggest

TABLE 7. Effect of drug resistance on performance of ZN staining MTD, and culture before and after start of ATC

M. tuberculosis	Test	No. positive/total (% positive)					
		Pre-ATC	Days 2 to 5	Days 5 to 15	Days 16 to 40	Days 41 to 80	Days 260 to 280
Sensitive[a]	ZN staining	14/27 (52)	2/11 (18)	1/14 (7)	0/16	0/10	0/15
	MTD	12/27 (44)	6/11 (55)	4/14 (29)	2/16 (13)	0/10	0/15
	Culture	24/27 (89)	2/11 (18)	3/14 (21)	0/16	0/10	0/15
Resistant[b]	ZN staining	10/15 (67)	4/10 (40)	0/12	0/11	0/10	0/10
	MTD	8/15 (53)	6/10 (60)	5/12 (42)	0/11	0/10	0/10
	Culture	14/15 (93)	10/10 (100)	3/12 (25)	0/11	1/10 (10)	0/10

[a] Sensitive to all first-line antituberculosis drugs.
[b] Resistant to one or more first-line antituberculosis drugs.

that they increase diagnostic sensitivity. The cumulative sensitivities of ZN staining, MTD, and the two tests combined are 64, 59, and 83%, respectively, if they are repeated at least twice during the first 15 days of therapy (Table 6). The value of a repeat MTD during the first 5 days of treatment was particularly evident, as a repeat test detected 29% more cases (10 of 35) than a single sample before ATC. This should discourage dangerous delays in starting ATC and may be useful to specialist centers admitting patients already started on ATC for unconfirmed TBM.

The impact of drug resistance upon bacterial clearance from the CSF is unknown, and this study could assess only the effect of streptomycin and isoniazid resistance. Evidence from pulmonary tuberculosis suggests that these two drugs, and in particular isoniazid, are responsible for the majority of bactericidal activity in the first few days of treatment (15). This evidence, together with known excellent penetration of isoniazid in CSF, suggests that resistance may have a detrimental effect upon outcome in TBM, but this remains unsubstantiated (22). This study suggests that resistance to isoniazid and/or streptomycin does affect CSF bacterial clearance: *M. tuberculosis* was cultured from all specimens after 2 to 5 days of ATC if the organism was resistant but from only 18% if the organism was sensitive ($P < 0.001$) (Table 7). The only organism to be cultured after 40 days of treatment was resistant to both drugs. The performances of ZN staining and MTD were not significantly different in patients with sensitive or resistant *M. tuberculosis*. Although these data suggest that isoniazid and/or streptomycin resistance reduces early bactericidal activity in the CSF, a larger study is required to define whether a positive ZN staining and/or MTD after the start of ATC can predict resistance and whether resistance to these agents worsens outcome. More useful still would be to use these methods to predict multidrug resistance, which carries a far worse prognosis and necessitates early intervention with second-line antituberculosis drugs. These studies are under way in our hospital.

The time and cost to obtain a result should also be considered when comparing CSF ZN staining with the MTD. These are especially important to busy laboratories in settings with poor resources, where the majority of TBM occurs. A CSF ZN staining takes approximately 1 h from lumbar puncture to result (15 min for centrifugation, 15 min for preparing the slide, and 30 min for microscopy), although more than 50% of positive slides are confirmed within the first 10 min of microscopy (21), and it can be performed without addition to basic laboratory equipment. It takes a minimum of 6 h to perform the CSF-modified MTD, regardless of the result, and the laboratory must have a luminometer (U.S. $12,500 [Gen-Probe, 2003]) and a sonicator (U.S. $495 [Gen-Probe, 2003]) and must purchase test kits that cost over U.S. $20 per test (50 tests/kit. U.S. $1,100/kit [Gen-Probe, 2003]).

In conclusion, this study shows that before the start of ATC, a careful search for AFB in the CSF is as good or better than the MTD detection of *M. tuberculosis* rRNA for the diagnosis of TBM. ZN staining is also faster and much less expensive. However, adding MTD to a careful microscopic examination of the CSF improves performance further and detects nearly 70% of cases before the start of treatment. Once treatment has been started, the MTD retains sensitivity longer than ZN staining or culture and may be more useful in settings where preadmission treatment with antituberculosis drugs is common. Resistance to one or more first-line antituberculosis drugs slows bacterial clearance from the CSF and significantly increases the likelihood of isolating *M. tuberculosis* from the CSF after 2 to 5 days of treatment. Repeated sampling after the start of treatment improves the sensitivity of all methods, particularly MTD. These results suggest that MTD alone offers no advantage over careful bacteriology before the start of treatment. However, performing a combination of these tests on repeated samples can improve sensitivity to greater than 80% while retaining high specificity. Nevertheless, the fatal consequences of delayed treatment demand a more sensitive single diagnostic test. Until less expensive, more sensitive assays are developed, the rapid diagnosis of TBM in most laboratories should depend on the meticulous and repeated search for AFB from a large volume (>6 ml) of CSF. Clinical algorithms may identify patients most likely to have TBM, with whom the most time should be spent (23).

ACKNOWLEDGMENTS

We thank the doctors and nurses of the Clinical Research Unit who cared for the patients and the staff of the Microbiology Laboratory, Hospital for Tropical Diseases.

The Wellcome Trust of Great Britain supported this study. Guy Thwaites is a Wellcome Trust Clinical Research Fellow.

REFERENCES

1. **Baker, C. A., C. P. Cartwright, D. N. Williams, S. M. Nelson, and P. K. Peterson.** 2002. Early detection of central nervous system tuberculosis with the Gen-Probe nucleic acid amplification assay: utility in an inner city hospital. Clin. Infect. Dis. **35:**339–342.
2. **Bonington, A., J. I. Strang, P. E. Klapper, S. V. Hood, A. Parish, P. J. Swift, J. Damba, H. Stevens, L. Sawyer, G. Potgieter, A. Bailey, and E. G. Wilkins.** 2000. TB PCR in the early diagnosis of tuberculous meningitis: evaluation of the Roche semi-automated COBAS Amplicor MTB test with reference to the manual Amplicor MTB PCR test. Tuber. Lung Dis. **80:**191–196.
3. **Bonington, A., J. I. Strang, P. E. Klapper, S. V. Hood, W. Rubombora, M. Penny, R. Willers, and E. G. Wilkins.** 1998. Use of Roche AMPLICOR *Mycobacterium tuberculosis* PCR in early diagnosis of tuberculous meningitis. J. Clin. Microbiol. **36:**1251–1254.
4. **Brienze, V. M., A. P. Tonon, F. J. Pereira, E. Liso, W. A. Tognola, M. A. dos Santos, and M. U. Ferreira.** 2001. Low sensitivity of polymerase chain reaction for diagnosis of tuberculous meningitis in southeastern Brazil. Rev. Soc. Bras. Med. Trop. **34:**389–393.
5. **Chedore, P., and F. B. Jamieson.** 2002. Rapid molecular diagnosis of tuberculous meningitis using the Gen-Probe amplified *Mycobacterium tuberculosis* direct test in a large Canadian public health laboratory. Int. J. Tuberc. Lung Dis. **6:**913–919.
6. **Donald, P. R., T. C. Victor, A. M. Jordaan, J. F. Schoeman, and P. D. van Helden.** 1993. Polymerase chain reaction in the diagnosis of tuberculous meningitis. Scand. J. Infect. Dis. **25:**613–617.
7. **Ehlers, S., R. Ignatius, T. Regnath, and H. Hahn.** 1996. Diagnosis of extrapulmonary tuberculosis by Gen-Probe amplified *Mycobacterium tuberculosis* direct test. J. Clin. Microbiol. **34:**2275–2279.
8. **Gamboa, F., J. M. Manterola, J. Lonca, B. Vinado, L. Matas, M. Gimenez, J. R. Manzano, C. Rodrigo, P. J. Cardona, E. Padilla, J. Dominguez, and V. Ausina.** 1997. Rapid detection of *Mycobacterium tuberculosis* in respiratory specimens, blood and other non-respiratory specimens by amplification of rRNA. Int. J. Tuberc. Lung Dis. **1:**542–555.
9. **Gamboa, F., J. M. Manterola, B. Vinado, L. Matas, M. Gimenez, J. Lonca, J. R. Manzano, C. Rodrigo, P. J. Cardona, E. Padilla, J. Dominguez, and V. Ausina.** 1997. Direct detection of *Mycobacterium tuberculosis* complex in nonrespiratory specimens by Gen-Probe amplified *Mycobacterium tuberculosis* direct test. J. Clin. Microbiol. **35:**307–310.
10. **Hosoglu, S., M. F. Geyik, I. Balik, B. Aygen, S. Erol, T. G. Aygencel, A. Mert, N. Saltoglu, I. Dokmetas, S. Felek, M. Sunbul, H. Irmak, K. Aydin, O. F. Kokglu, H. Ucmak, M. Altindus, and M. Loeb.** 2002. Predictors of outcome in patients with tuberculous meningitis. Int. J. Tuberc. Lung Dis. **6:**64–70.
11. **Kaneko, K., O. Onodera, T. Miyatake, and S. Tsuji.** 1990. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction (PCR). Neurology **40:** 1617–1618.

12. **Kennedy, D. H., and R. J. Fallon.** 1979. Tuberculous meningitis. JAMA **241:**264–268.
13. **Lang, A. M., J. Feris-Iglesias, C. Pena, J. F. Sanchez, L. Stockman, P. Rys, G. D. Roberts, N. K. Henry, D. H. Persing, and F. R. Cockerill III.** 1998. Clinical evaluation of the Gen-Probe amplified direct test for detection of *Mycobacterium tuberculosis* complex organisms in cerebrospinal fluid. J. Clin. Microbiol. **36:**2191–2194.
14. **Medical Research Council.** 1948. Streptomycin treatment of tuberculous meningitis. Br. Med. J. **i:**582–597.
15. **Mitchison, D. A.** 2000. Role of individual drugs in the chemotherapy of tuberculosis. Int. J. Tuberc. Lung Dis. **4:**796–806.
16. **Pai, M., L. L. Flores, N. Pai, A. Hubbard, L. W. Riley, and J. M. Colford.** 2003. Diagnostic accuracy of nucleic acid amplification tests for tuberculous meningitis: a systematic review and meta-analysis. Lancet Infect. Dis. **3:**633–643.
17. **Pfyffer, G. E., P. Kissling, E. M. Jahn, H. M. Welscher, M. Salfinger, and R. Weber.** 1996. Diagnostic performance of amplified *Mycobacterium tuberculosis* direct test with cerebrospinal fluid, other nonrespiratory, and respiratory specimens. J. Clin. Microbiol. **34:**834–841.
18. **Shankar, P., N. Manjunath, K. K. Mohan, K. Prasad, M. Behari, Shriniwas, and G. K. Ahuja.** 1991. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. Lancet **337:**5–7.
19. **Stewart, S. M.** 1953. The bacteriological diagnosis of tuberculous meningitis. J. Clin. Pathol. **6:**241–242.
20. **Thwaites, G., T. T. Chau, N. T. Mai, F. Drobniewski, K. McAdam, and J. Farrar.** 2000. Tuberculous meningitis. J. Neurol. Neurosurg. Psych. **68:**289–299.
21. **Thwaites, G., T. T. H. Chau, J. J. Farrar.** 2004. Improving the bacteriological diagnosis of tuberculous meningitis. J. Clin. Microbiol. **42:**378–379.
22. **Thwaites, G. E., T. T. Chau, M. Caws, N. H. Phu, L. V. Chuong, D. X. Sinh, F. Drobniewski, N. J. White, C. M. Parry, and J. J. Farrar.** 2002. Isoniazid resistance, mycobacterial genotype and outcome in Vietnamese adults with tuberculous meningitis. Int. J. Tuberc. Lung Dis. **6:**865–871.
23. **Thwaites, G. E., T. T. Chau, K. Stepniewska, N. H. Phu, L. V. Chuong, D. X. Sinh, N. J. White, C. M. Parry, and J. J. Farrar.** 2002. Diagnosis of adult tuberculous meningitis by use of clinical and laboratory features. Lancet **360:**1287–1292.