Tuberculosis Diagnostics in the New Millennium: Role in TB Identification and Control

Guest Editors: Soumitesh Chakravorty, Catharina Boehme, and Jongseok Lee
Tuberculosis Diagnostics in the New Millennium: Role in TB Identification and Control
Tuberculosis Diagnostics in the New Millennium: Role in TB Identification and Control

Guest Editors: Soumitesh Chakravorty, Catharina Boehme, and Jongseok Lee
Editorial Board

J. K. Actor, USA
A. S. Apt, Russia
M. S. Baird, UK
M. R. Barer, UK
Christos Chouaid, France
Luis E Cuevas, UK
Karl Drlica, USA
Brian Eley, South Africa
Carlo Garzelli, Italy
E. A. Graviss, USA
Jacques Grosset, USA
A. S. Hammond, Mali
S. E. Hasnain, India
R. L. Hunter, USA
Juraj Ivanyi, UK
Vincent Jarlier, France
P. R. Klatser, The Netherlands
Jos R. Lapa e Silva, Brazil
Roy Mugerwa, Uganda
T. Ottenhoff, The Netherlands
David C. Perlman, USA
Nalin Rastogi, France

Giovanna Riccardi, Italy
Menico Rizzi, Italy
W. N. Rom, USA
Euzenir N. Sarno, Brazil
Sarman Singh, India
Jeffrey R. Starke, USA
Isamu Sugawara, Japan
T. E. Tupasi, Philippines
Robert S. Wallis, USA
## Contents

**Tuberculosis Diagnostics in the New Millennium: Role in TB Identification and Control,** Soumitesh Chakravorty, Catharina Boehme, and Jongseok Lee  
Volume 2012, Article ID 768603, 2 pages

**Pouched Rats’ Detection of Tuberculosis in Human Sputum: Comparison to Culturing and Polymerase Chain Reaction,** Amanda Mahoney, Bart J. Weetjens, Christophe Cox, Negussie Beyene, Klaus Reither, George Makingi, Maureen Jubitana, Rudovick Kazwala, Godfrey S. Mfinanga, Amos Kahwa, Amy Durgin, and Alan Poling  
Volume 2012, Article ID 716989, 5 pages

**The PCR-Based Diagnosis of Central Nervous System Tuberculosis: Up to Date,** Teruyuki Takahashi, Masato Tamura, and Toshiaki Takasu  
Volume 2012, Article ID 831292, 17 pages

**Maintenance of Sensitivity of the T-SPOT.TB Assay after Overnight Storage of Blood Samples,** Dar es Salaam, Tanzania, Elizabeth A. Talbot, Isaac Maro, Katherine Ferguson, Lisa V. Adams, Lillian Mtei, Mecky Matee, and C. Fordham von Reyn  
Volume 2012, Article ID 345290, 4 pages

**The Use of Interferon Gamma Release Assays in the Diagnosis of Active Tuberculosis,** Silvan M. Vesenbeckh, Nicolas Schönfeld, Harald Mauch, Thorsten Bergmann, Sonja Wagner, Torsten T. Bauer, and Holger Rüssmann  
Volume 2012, Article ID 768723, 4 pages

**Comparison of Overnight Pooled and Standard Sputum Collection Method for Patients with Suspected Pulmonary Tuberculosis in Northern Tanzania,** Stellah G. Mpagama, Charles Mtabho, Solomon Mwaigwisya, Liberate J. Mleoh, I Marion Sumari-de Boer, Scott K. Heysell, Eric R. Houpt, and Gibson S. Kibiki  
Volume 2012, Article ID 128057, 5 pages
Tuberculosis (TB) continues to remain a significant threat even as we have moved into the second decade of the 21st century. As a matter of fact TB has assumed an even more ominous stance with the emergence of totally drug resistant or TDR *Mycobacterium tuberculosis* (*M. tb*) strains which are virtually untreatable. Unfortunately, there has been no new effective vaccine against tuberculosis, and in spite of introduction of several new drugs like bedaquiline, delamanid, PA824, or SQ109 and a new generation of fluoroquinolones like gatifloxacin and moxifloxacin, they are yet to be involved in the current routine antituberculosis regimen, though clinical trials for combinatorial therapy along with currently used drugs are underway. The emergence of resistance against these new classes of drugs is also a likely possibility which will result in the same problems in the future with newer group of drug-resistant strains, as we are facing today with the multidrug resistant (MDR) and extensively drug-resistant (XDR) strains. Under these circumstances, rapid and definitive molecular diagnostics for effective intervention and treatment of TB patients is a cornerstone for appropriate disease control and eradication. Recently, a lot of attention has been devoted towards rapid TB diagnostics especially those which enable rapid drug susceptibility testing (DST) to break free from the century long dependence on smear microscopy and culture methods, which are frustratingly insensitive and time consuming, respectively. WHO has recently recommended automated liquid culture systems, line probe assays, and the Xpert MTB/RIF tests which allow faster DST results and highly sensitive detection of *M. tb* from clinical samples. These assays signal towards the emerging era of rapid and decisive tests employing new generation of molecular and microbiological methods. Against this exciting backdrop of the emerging trends and innovations in TB diagnostic techniques, the special edition of *Tuberculosis Research and Treatment* focuses on tuberculosis diagnostics in the new millennium, role in TB identification and control, which represents a perspective on the emerging trends in the next of generation TB diagnostics. From unconventional approaches like using sputum sniffing pouched rats to the latest immunodiagnostics like interferon gamma release assays (IGRAs), a detailed overview on the current PCR-based techniques of diagnosis of TB meningitis, as well as importance of the methods of sample collection for accurate diagnosis of TB, are dealt with in this issue. One elegant study by A. Mahoney et al. examines the utility of using pouched rats for detection of tuberculosis as an adjunct to smear microscopy in resource poor countries. The paper by T. Takahashi et al. explores the current PCR-based approaches to diagnose one of the most deadly forms of extrapulmonary TB, TB meningitis, and describes a novel “wide range quantitative nested real-time PCR” assay. Two papers by E. A. Talbot et al. and S. M. Vesenebeckl et al. deal with one of the most widely used immunodiagnosticstics for TB, namely, IGRA and its applicability in TB diagnostics. One paper examined its utility in overnight-stored blood samples, and a second study assessed the accuracy of the recommended cut-off values for diagnosing active TB, in which both have important implications on utility and applicability of IGRA. The paper by Mpagama et al. brings forth the importance of using overnight-pooled sputum in enhancing the sensitivity
and shortening the time of detection using the BACTEC MGIT system. This special edition on the current scenario of TB diagnostics will serve as a concise but comprehensive spectrum of the different approaches aimed at faster and more definitive detection of TB disease and its control.

Soumitesh Chakravorty
Catharina Boehme
Jongseok Lee
Clinical Study

Pouched Rats’ Detection of Tuberculosis in Human Sputum: Comparison to Culturing and Polymerase Chain Reaction

Amanda Mahoney,1, 2 Bart J. Weetjens,2 Christophe Cox,2 Negussie Beyene,2 Klaus Reither,3, 4 George Mavingi,5 Maureen Jubitana,2 Rudovick Kazwala,5 Godfrey S. Mfinanga,6 Amos Kahwa,6 Amy Durgin,1, 2 and Alan Poling1, 2

1 Department of Psychology, Western Michigan University, Kalamazoo, MI 49008-5200, USA
2 Tuberculosis Research, Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling (APOPO), Morogoro, Tanzania
3 Tuberculosis Research, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland
4 TB Research and Training Center, Ifakara Health Institute, Bagamoyo, Tanzania
5 Department of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania
6 Clinical Research Laboratory, National Institute for Medical Research, Dar es Salaam, Tanzania

Correspondence should be addressed to Amanda Mahoney, amanda.mahoney@apopo.org

Received 22 December 2011; Revised 16 May 2012; Accepted 16 May 2012

Academic Editor: Soumitesh Chakravorty

Copyright © 2012 Amanda Mahoney et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Setting. Tanzania. Objective. To compare microscopy as conducted in direct observation of treatment, short course centers to pouched rats as detectors of Mycobacterium tuberculosis. Design. Ten pouched rats were trained to detect tuberculosis in sputum using operant conditioning techniques. The rats evaluated 910 samples previously evaluated by smear microscopy. All samples were also evaluated through culturing and multiplex polymerase chain reaction was performed on culture growths to classify the bacteria. Results. The patientwise sensitivity of microscopy was 58.0%, and the patient-wise specificity was 97.3%. Used as a group of 10 with a cutoff (defined as the number of rat indications to classify a sample as positive for Mycobacterium tuberculosis) of 1, the rats increased new case detection by 46.8% relative to microscopy alone. The average samplewise sensitivity of the individual rats was 68.4% (range 61.1–73.8%), and the mean specificity was 87.3% (range 84.7–90.3%). Conclusion. These results suggest that pouched rats are a valuable adjunct to, and may be a viable substitute for, sputum smear microscopy as a tuberculosis diagnostic in resource-poor countries.

1. Introduction

A major hurdle in combating tuberculosis (TB) is diagnosing the disease in resource-poor countries. Sputum smear microscopy, the technique typically used, is relatively slow and characteristically has high specificity but low sensitivity [1, 2]; therefore, the international medical community has prioritized developing a quick, accurate, and affordable alternative diagnostic. In an attempt to develop one, researchers recently have investigated the use of scent-detecting pouched rats (Cricetomys gambianus) as a TB diagnostic. An initial proof of principle investigation [3] revealed that pouched rats trained through operant conditioning procedures could detect TB in human sputum, and three subsequent studies, involving a total of over 20,000 patients, showed that using the rats in second-line screening of sputum samples initially screened by smear microscopy at direct observation of treatment—short course (DOTS) centers in Tanzania increased new case detections by 31.4% [4], 44% [5], and 42.8% [6].

These results are promising, but the accuracy of Cricetomys in detecting TB has not been extensively evaluated relative to an established reference standard. Culturing is considered the “gold standard” for TB detection [2], and Weetjens et al. [3] reported the results of a study in which two rats, Mandela and Kingston, evaluated 817 sputum samples also evaluated by culturing, which revealed 67 TB-positive samples. Sensitivity relative to culturing for both rats was 73.1%, while specificity was 97% and 97.8% for Mandela and Kingston, respectively. In an attempt to provide more
comprehensive information regarding pouched rats’ TB-detection accuracy relative to the best available and affordable method, this experiment evaluated 10 rats’ performance compared to culture in combination with Multiplex PCR.

2. Method

2.1. Subjects and Materials. Ten adult Cricetomys obtained from our breeding colony, 5 males and 5 females, evaluated all sputum samples. The animals were housed and maintained as detailed elsewhere [3, 7]. Ethical clearance to conduct the research was obtained from the Tanzanian National Institute for Medical Research. Some of the rats had been used in previous studies and all of the rats had been evaluating sputum samples for TB for at least one year.

Testing was conducted in a chamber 205 cm long, 55 cm wide, and 55 cm high with clear plastic walls and ceiling and a stainless steel floor. Ten holes with sliding lids 2.5 cm in diameter were spaced equidistance apart along the centerline of the chamber floor’s long axis. Pots containing sputum were placed beneath the holes for the rats to evaluate. Edible reinforcers (rewards), consisting of a mixture of mashed banana with ground rodent diet pellets, were delivered through a plastic syringe through feeding holes.

2.2. Collection of Sputum Samples. Sputum samples were collected weekly from eight DOTS centers in Dar es Salaam and Morogoro, Tanzania, using World Health Organization (WHO) recommended sputum containers. Direct smear microscopy after Ziehl-Neelsen staining was conducted at the DOTS centers prior to collection of the samples. Samples of less than 2 mL were excluded to ensure that there was sufficient volume for culturing and rat evaluation. In all, 910 samples from 456 patients (two from each of 454, one from each of two) were evaluated. Before evaluation by rats, an aliquot was taken from each sample for culture purposes, and then sterile phosphate buffered saline solution (5 mL) was added to each sputum sample and microorganisms were inactivated by heating the sample at 90°C for 30 min [8]. The samples were then frozen at −20°C until the day of evaluation (up to seven days). Though there is some controversy surrounding the cellular impact of freezing and thawing sputum, past research suggests that samples may be kept frozen without significant alteration of cell quality or cell counts [9]. Furthermore, data collected internally suggest that the rats’ performance is unaffected by the freezing procedures employed. Samples were thawed four times for the purpose of this study: once on the day of collection, once to take aliquots for culture, once to evaluate the sputum quantity and add buffer, and once on the day of evaluation by the rats.

2.3. Rats’ Evaluation of Samples. Prior to this study, the rats were trained to detect TB as detailed elsewhere [3, 7]. In the present study, each rat evaluated each sample twice, in a different order, across 13 sessions. In each session, 63 samples found negative by microscopy at DOTS centers and seven samples found positive were presented to the rats. The seven positive samples served as reinforcement opportunities to maintain the rats’ indications while the remaining samples were categorized as “unknown”. During the sessions, the experimenter opened each hole in the cage as the rat passed over and sniffed. When the rat paused for 5 s (i.e., emitted an indicator response), the experimenter informed a data collector who then stated whether the sample was smear positive according to DOTS-center microscopy. If the rat made an indicator response above a smear-positive sample, the experimenter sounded a click and delivered food, after which the rat then moved to the next hole to continue evaluations. If the rat emitted an indicator response at a smear-negative sample, which is considered an unknown sample, the experimenter closed the hole but did not sound a click or present food.

2.4. Data Analysis of Rat Results. Following evaluations, the rats’ performance was assessed relative to the results of culture with M. tuberculosis Multiplex PCR. Sensitivity and specificity were calculated for the group of 10 rats, and thus the criterion for counting a rat-positive indication could be an indication on either or both sample presentations by one rat, ten rats, or any number of rats in between, which are referred to hereafter as cutoffs 1–10. At a cutoff of 3, for example, a sample was deemed rat positive if three or more rats indicated it; samples indicated by only 2, 1, or 0 rats were deemed negative.

2.5. Culturing and PCR. Culturing was conducted in accordance with an established and recommended procedure for culturing sputum samples on Lowenstein-Jensen (LJ) solid media (WHO Guidelines on Standard Operating Procedures for Microbiology, Tuberculosis, WHO Regional Office for Southeast Asia, 2006). Decontaminated samples were inoculated onto different tubes of Lowenstein-Jensen solid media, one with pyruvate and the other with glycerol. The tubes were incubated at 37°C and inspected weekly for eight weeks. Media on which microbial growth was observed were scraped, stained by the ZN method, and analyzed by light microscopy. Each specimen which exhibited either AFB-positive culture material or characteristic bacterial growth was further analyzed by Multiplex PCR.

In the first step of PCR [10], Multiplex PCR genus typing was conducted to identify species belonging to the Mycobacterium genus. This genotyping distinguished species belonging to the Mycobacterium tuberculosis complex (MTC), specifically M. bovis, M. africanum, M. tuberculosis, and M. microti, from nontuberculous mycobacteria (M. avium, M. intracellulare, and others). All bacterial suspensions or DNA extracts containing MTC were subjected to another PCR, deletion typing. This procedure differentiated bacteria that were M. tuberculosis, M. bovis, or M. africanum.

3. Results

All sputum samples were classified as positive or negative for M. tuberculosis by microscopy at the DOTS centers, culturing (with PCR as appropriate), and rats’ evaluation.
Culture-positive samples were those in which characteristic growth was stained with a Ziehl-Neelsen stain and the presence of acid-fast bacilli confirmed. A sample was further considered PCR-positive if, following amplification, the presence of acid-fast bacilli confirmed. A sample was considered rat positive if at least one rat indicated.

Table 1: Samples classified as *M. tuberculosis* and non-*M. tuberculosis* by Multiplex PCR.

<table>
<thead>
<tr>
<th></th>
<th><em>M. tuberculosis</em></th>
<th>Non-<em>M. tuberculosis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PCR+</td>
<td>129</td>
<td>13</td>
</tr>
<tr>
<td>ZN+ glycerol/pyruvate</td>
<td>(102/87)</td>
<td>(2/13)</td>
</tr>
<tr>
<td>Rat+</td>
<td>109</td>
<td>10</td>
</tr>
<tr>
<td>Smear+ (DOTS)</td>
<td>86</td>
<td>7</td>
</tr>
</tbody>
</table>

*A sample was considered rat positive if at least one rat indicated.*

Table 2: Sample-wise and patient-wise sensitivity and specificity at rat agreements cutoffs 1–10.

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>Samplewise</th>
<th>Patientwise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>1</td>
<td>84.50</td>
<td>64.00</td>
</tr>
<tr>
<td>2</td>
<td>81.40</td>
<td>75.70</td>
</tr>
<tr>
<td>3</td>
<td>76.00</td>
<td>81.80</td>
</tr>
<tr>
<td>4</td>
<td>74.40</td>
<td>86.60</td>
</tr>
<tr>
<td>5</td>
<td>68.20</td>
<td>89.40</td>
</tr>
<tr>
<td>6</td>
<td>66.70</td>
<td>91.90</td>
</tr>
<tr>
<td>7</td>
<td>65.10</td>
<td>93.30</td>
</tr>
<tr>
<td>8</td>
<td>62.80</td>
<td>95.50</td>
</tr>
<tr>
<td>9</td>
<td>58.90</td>
<td>96.90</td>
</tr>
<tr>
<td>10</td>
<td>53.50</td>
<td>98.10</td>
</tr>
</tbody>
</table>

*Relative to Multiplex PCR.*

4. Discussion

In this study, 10 adult *Cricetomys* evaluated 910 sputum samples collected from patients suspected for tuberculosis. Weetjens et al. [3] previously reported that each of two pouched rats yielded a sensitivity of 73.1% relative to culturing, and their specificities were 97% and 97.8%. In the present study, which included Multiplex PCR, somewhat lower values were obtained where the mean individual sample-wise sensitivity of 10 rats relative to culture/PCR was 68.4%, and the mean specificity was 87.3%. Nonetheless, each rat’s sensitivity exceeded that of ZN smear microscopy performed as part of routine TB screening at DOTS centers, although their specificity was lower. Because the rats can evaluate samples quickly, it is tenable to have several of them evaluate each sputum sample, and this has been done in studies examining their use in second-line screening of samples initially evaluated by ZN microscopy [4–6]. For example, Poling et al. [5] used a cutoff of 2 of 10 rats for identifying a sample as TB-positive. The present data suggest that this is a reasonable criterion in terms of balancing sensitivity and specificity, which were 81.4% and 75.7% when it was used in the present study. Similar values were obtained with cutoffs of 3 of 10 and 4 of 10. With a cutoff of 2, the rats as a group detected 66 of 81 patients found...
Table 3: Average sensitivity and specificity for 12 groups of 4, 3, and 2 rats.

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>4 Rats</th>
<th>3 Rats</th>
<th>2 Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>1</td>
<td>79.9</td>
<td>73.8</td>
<td>77.2</td>
</tr>
<tr>
<td>2</td>
<td>71.5</td>
<td>86.4</td>
<td>68.8</td>
</tr>
<tr>
<td>3</td>
<td>64.8</td>
<td>92.0</td>
<td>60.6</td>
</tr>
<tr>
<td>4</td>
<td>57.8</td>
<td>96.2</td>
<td></td>
</tr>
</tbody>
</table>

*Relative to culture/PCR.

Table 4: Patient-wise smear microscopy and average rat reference results.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Pos predictive value</th>
<th>Neg predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture/PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correct/total samples (%)</td>
<td>47/81 (58.0)</td>
<td>407/409 (99.5)</td>
<td>.96</td>
<td>.92</td>
</tr>
<tr>
<td>95% confidence interval (CI)</td>
<td>46.6–68.7</td>
<td>98–99.9</td>
<td>.84–.99</td>
<td>.89–.95</td>
</tr>
<tr>
<td>Rat (average of 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correct/total samples (%)</td>
<td>57.1/81 (70.5)</td>
<td>329.1/409 (80.5)</td>
<td>.42</td>
<td>.93</td>
</tr>
<tr>
<td>95% CI</td>
<td>68.2–72.8</td>
<td>78.4–82.6</td>
<td>.33–.50</td>
<td>.89–.96</td>
</tr>
</tbody>
</table>

*Smear microscopy conducted at DOTS centers.

to be TB-positive by culturing/PCR, whereas DOTS centers’ microscopy detected 47 of these patients. Therefore, had the rats been used in second-line screening as in prior studies [5, 6] and their results verified, they would have increased new-case detections by 40.4%.

Results revealed 57 culture-negative and three culture-positive, but PCR-negative samples indicated by six or more rats. To clarify the status of these samples, an internal test was done on all of these plus 100 randomly-selected rat-negative samples using the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) [11]. Analysis by the GeneXpert revealed 25 positive samples and 23 positive patients that had previously been classified as negative by culture or Multiplex PCR, bringing the combined Multiplex PCR and MTB/RIF positive samples to 154 and positive patients to 98. The test reclassified 18 of the 60 rat-positive samples and 7 of the 100 rat-negative samples. An analysis was conducted including these reclassified participants and revealed a patient-wise sensitivity of microscopy at DOTS centers of 48% and specificity of 98.3%. For the rats, patient-wise sensitivity was 67% (range 62.2–72.5%) while their mean specificity was 93.5% (range 91.1–95.3%). Due to the costs of the cartridges required for the GeneXpert, it was not possible to evaluate all samples and, to avoid a possible bias, these data were not incorporated into the main results. These additional data suggest that the specificity of the rats may be higher than that suggested by the comparison to culture and, to test this possibility these findings, a study is underway that will thoroughly evaluate the rats relative to MTB/RIF.

In prior studies, a second microscopy was used to verify the status of DOTS-negative, rat-positive samples, but such confirmation is weak. The rats identify as positive a relatively high number of TB-negative samples; however, relying on microscopy alone allows a substantial number of patients with TB to go undetected. A better procedure would be to use the GeneXpert to confirm the status of rat-positive, smear-negative samples. Confirmation of samples in this way is likely to reveal a substantial number of TB-positive patients missed with the present procedure and thus slow the spread of transmission. This benefit would seemingly justify the financial cost of using the GeneXpert.

In addition to finding TB-positive patients overlooked by microscopy, the rats may potentially yield savings in time and cost. The rats are faster in evaluation than a lab technician, but require that the samples be transported and processed. These steps are completed with large batches of samples and it is important that future studies clearly demonstrate the cost-efficiency of these procedures relative to microscopy and investigate potential savings in costs and time. Prospectively, should the rats be called upon as a first-line screening tool, research on the MTB/RIF assay indicates that its high cost may limit its global utility [12], and the rats seem particularly well suited to work in conjunction with this technology to reduce costs. The rats screen samples quickly and, if used in areas with prevalence similar to that in which they have been tested, will reduce the number of patients in need of followup. The outcome of such a setup depends largely upon the number of rats used. Extrapolating from the current results, one could expect to recheck about 10% of samples if one rat is used and recheck about 45% of samples if 10 rats are used. The ideal number, as illustrated in Table 4, is probably between 2 and 4 rats as sensitivity remains relatively high while the false alarm rate improves with fewer rats.

A significant limitation of the present study is that no clinical data were available for comparison. TB-positive patients evaluated by TB specialists are more likely to be identified than those diagnosed by smear results alone [13], and so a study is underway at APOPO that will incorporate clinical data. A second limitation is that the HIV status of patients was not available to us; therefore, the
sensitivity and specificity of DOTS microscopy and the rats could not be compared in HIV-positive and HIV-negative patients. Further research in this area is planned to make the comparison and to evaluate the value of the rats in detecting TB in children.

5. Conclusions

In this study, DOTS microscopy found 58% of the culture/Multiplex PCR positive patients, which is similar to results found in past studies [2, 11], compared to an average of 70.5% of positive patients found by individual rats. The results presented herein, combined with previously published operational data, demonstrate that the rats are faster than smear microscopy as commonly practiced and can identify more TB-positive patients. There is now substantial evidence that when used for second-line screening, *Cricetomys* can have a large positive impact on TB detection and public health in high-incidence areas, such as sub-Saharan Africa, although future research is necessary to refine training techniques to identify the applications for which the rats are best suited and to ascertain their per-case-detected cost relative to alternative diagnostics.

Authors’ Contribution

This work was carried out in collaboration among all authors listed. C. Cox, B. J. Weetjens, A. Poling, G. Makingi, and A. Mahoney conceptualized this research and provided critical intellectual content. R. Kazwala, G. S. Mfinanga, and K. Reither provided expertise on the laboratory operations necessary for carrying out this experiment, contributed to the conceptualization of this research, and participated in the discussion on presentation of the results. A. M. Mahoney, N. Beyene, M. Jubitana, D. Kuipers, and A. Durgin oversaw the laboratory experiments, analyzed the data, and interpreted the results. A. Poling, A. Mahoney, and N. Beyene wrote the paper. All authors have contributed to, seen, and approved this paper.

Acknowledgments

The authors wish to acknowledge the trainers, secretaries, and laboratory technicians in the TB laboratory at APOPO for their hard work throughout this experiment. This work would not have been possible without their support. Financial support for this work was provided by the UBS Optimus Foundation. The authors declare that they have no competing interests, and specifically have no competing interests with respect to the use of the Cepheid GeneXpert.

References

The PCR-Based Diagnosis of Central Nervous System Tuberculosis: Up to Date

Teruyuki Takahashi,1 Masato Tamura,1,2 and Toshiaki Takasu1,2

1 Department of Neurology, Nagaoka-Nishi Hospital Mitsugohya-machi, 371-1 Nagaoka City, Niigata, Japan
2 Division of Neurology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan

Correspondence should be addressed to Toshiaki Takasu, ttakasu@vesta.ocn.ne.jp

Received 2 December 2011; Accepted 14 February 2012

Academic Editor: Soumitesh Chakravorty

Copyright © 2012 Teruyuki Takahashi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Central nervous system (CNS) tuberculosis, particularly tuberculous meningitis (TBM), is the severest form of Mycobacterium tuberculosis (M.Tb) infection, causing death or severe neurological defects in more than half of those affected, in spite of recent advancements in available anti-tuberculosis treatment. The definitive diagnosis of CNS tuberculosis depends upon the detection of M.Tb bacilli in the cerebrospinal fluid (CSF). At present, the diagnosis of CNS tuberculosis remains a complex issue because the most widely used conventional “gold standard” based on bacteriological detection methods, such as direct smear and culture identification, cannot rapidly detect M.Tb in CSF specimens with sufficient sensitivity in the acute phase of TBM. Recently, instead of the conventional “gold standard”, the various molecular-based methods including nucleic acid amplification (NAA) assay technique, particularly polymerase chain reaction (PCR) assay, has emerged as a promising new method for the diagnosis of CNS tuberculosis because of its rapidity, sensitivity and specificity. In addition, the innovation of nested PCR assay technique is worthy of note given its contribution to improve the diagnosis of CNS tuberculosis. In this review, an overview of recent progress of the NAA methods, mainly highlighting the PCR assay technique, was presented.

1. Introduction

Central nervous system (CNS) disease caused by Mycobacterium tuberculosis (M.Tb), particularly tuberculous meningitis (TBM), is uncommon and accounts for approximately 1% of all tuberculosis cases in the United States [1, 2]. CNS tuberculosis is the severest form of M.Tb infection, causing death or severe neurological defects in more than half of those affected, in spite of recent advancements in available antituberculosis treatment (ATT) [1–5]. In addition, owing to an increasing number of immunocompromised hosts caused by the prevalence of AIDS, increasing numbers of older people, the wider use of immunosuppressive agents, and other factors, TBM remains a serious clinical and social problem [1–5]. Owing to its relative rarity and the wide spectrum of its neurological symptoms, CNS tuberculosis remains a formidable diagnostic challenge [1–5]. In TBM, accurate and rapid diagnosis and early treatment for tuberculosis are the most important factors with regard to the prognosis and the prevention of long-term neurological sequelae [1–5]. However, the conventional “gold standard” based on bacteriological detection methods of M.Tb, including the direct smear examination for acid-fast bacilli (AFB) and culture identification, is inadequate for early diagnosis, owing to the poor sensitivity or the long time required (4–8 weeks) for cultures [1–5].

Recently, the detection of M.Tb DNA in the cerebrospinal fluid (CSF) through the use of various molecular-based methods, including nucleic acid amplification (NAA) assay technique, particularly polymerase chain reaction (PCR) assay, has emerged as a promising new method for the diagnosis of CNS tuberculosis because of its rapidity, sensitivity, and specificity [2–42]. Many investigators have reported on the usefulness of PCR assay for the detection of M.Tb DNA in CSF, although the sensitivity of PCR assay has significant discrepancies (65–83%) between each type of measuring methods and different laboratories [2–42]. Moreover, nested PCR assay has been reported as a prominent method for detecting M.Tb DNA in CSF.
This new method drastically increases the sensitivity and specificity of DNA amplification compared with conventional single-step PCR [3, 4, 17, 19, 24–27, 32]. However, the nested PCR assay using CSF samples has yet to be widely used in TBM diagnosis owing to its laborious and time-consuming procedure, which carries a high risk of cross-contamination [3, 4, 17, 19, 24–27, 32]. Currently, real-time PCR assay is applied in routine diagnostic laboratory testing [33, 38, 40–42]. In addition to conventional qualitative analysis, real-time PCR assay makes it possible to perform accurate quantitative analyses with a high degree of reproducibility [33, 38, 40–42].

In this paper, the authors highlight the recent advancement of NAA assay techniques, in particular PCR assay and provide an overview of the current issues and evolution of diagnosis and clinical aspects of CNS tuberculosis.

2. The Global Epidemiologic Burden of Tuberculosis

In 2007, the World Health Organization (WHO) estimated that 9.27 million new cases (139/100,000 population) of active tuberculosis occur annually, resulting in an estimated 1.6 million deaths per year [1, 2]. Tuberculosis remains a worldwide burden, with a large majority of new active tuberculosis cases occurring in underdeveloped and developing countries [1, 2]. In fact, India, China, Indonesia, Nigeria, and South Africa rank first to fifth in the total number of incident cases of tuberculosis [1, 2]. In 80% of new tuberculosis cases, social and demographic factors such as poverty, overcrowding, malnutrition, and a compromised immune system play a major role in the worldwide epidemic, while the remaining 20% of tuberculosis cases are associated with HIV in sub-Saharan Africa [1, 2]. Among the 9.27 million new tuberculosis cases in 2007, an estimated 1.37 million (14.8%) were HIV positive [1, 2].

CNS infection is one of the severest forms of tuberculosis [1–5]. In a large-scale epidemiological study of extrapulmonary tuberculosis in the United States, CNS involvement was noted in 5 to 10% of extrapulmonary tuberculosis cases, with more recent CDC data in 2010 indicating that 5.5% of extrapulmonary cases involve CNS tuberculosis (=1.2% of total tuberculosis cases) [43–46]. In the largest prospective epidemiological study of CNS tuberculosis, the incidence of developing CNS tuberculosis was approximately 1.0% among 82,764 tuberculosis cases from 1970 to 2001 in a Canadian cohort [1, 2, 43]. However, despite an overall decrease in the total number of tuberculosis cases in advanced nations such as the United States, a gradual and continuous increase in the proportion of extrapulmonary tuberculosis cases has been reported [1, 2, 43–46]. This increase has been mainly attributed to the recent increase of immunocompromised patients and the HIV/AIDS epidemic [1, 2, 43–46]. In addition, although the overall population-based mortality rate from tuberculosis is low and decreasing, several studies have shown that mortality rates are substantially higher in patients with several forms of extrapulmonary tuberculosis, including CNS tuberculosis or TBM and disseminated disease [1, 2, 43–46].

Several risk factors for CNS tuberculosis have been identified. Both age (children > adults) and HIV-coinfected patients are at high risk for developing CNS tuberculosis [1, 2, 44]. Other risk factors include malnutrition, recent measles in children, alcoholism, malignancies, the use of immunosuppressive agents in adults, and disease prevalence in the community [1, 2]. Several studies in developed countries have also identified that foreign-born individuals (individuals born outside of developed countries) are overrepresented among CNS tuberculosis cases [1, 2, 44–46].

3. Current Issues for Diagnosis in CNS Tuberculosis

At present, the diagnosis of CNS tuberculosis remains a complex issue because the most widely used conventional bacteriological detection methods, such as direct smear for AFB and culture for M.Tb, cannot rapidly detect M.Tb in CSF specimens with sufficient sensitivity in the acute phase of TBM [2–42]. Rapid and accurate diagnosis in the acute phase of CNS tuberculosis and early starting ATT are the most important factors with regard to the prognosis and the prevention of long-term neurological sequelae [2–5]. The poor sensitivity and often delayed results from the conventional “gold standard” based on microbiological techniques in the traditional TBM diagnosis underscore the need for a more sensitive, rapid, and accurate diagnostic method in clinical practice [2–5]. Several molecular-based methods, often drawn from successful techniques used for the diagnosis of tuberculosis in respiratory specimens, have been evaluated for their applicability in the diagnosis of CNS tuberculosis. These techniques include commercially available NAA methods and other PCR-based methods [2–42]. In addition, the use of neuroradiographic techniques such as magnetic resonance imaging (MRI) has prominently improved the diagnostic accuracy of TBM and tuberculomas [2, 47–49]. Recently, the role of neuroradiographic techniques in the evaluation of CNS tuberculosis has been reviewed in various reports [1–5, 47–49]. Commonly identified neuroradiological features of TBM include basal meningeal enhancement, hydrocephalus, and infarctions in the supratentorial brain parenchyma and brain stem [2, 47–49]. Moreover, tuberculomas are generally defined as low- or high-intensity, round or lobulated masses with irregular walls and show homogeneous or ring enhancement after the administration of contrast [2, 47–49]. They occur as solitary or multiple nodules and are typically found in the frontal and parietal lobes [2, 47–49]. However, the differential diagnosis of tuberculomas and other intracranial focal massive lesions such as fungal granulomas is difficult when using only neuroradiographic techniques [2]. Therefore, the combination of molecular-based techniques and neuroradiographic techniques is regarded as a promising and powerful diagnostic tool for TBM and tuberculomas, instead of neuroradiographic techniques with or without classical bacteriological detection methods [2].
4. Recent Advancement of PCR Assay Technique

The NAA methods for *M. Tb* are diagnostic techniques to demonstrate the presence of tubercle bacilli by the extraction and amplification of DNA or RNA of *M. Tb* from clinical specimens such as sputum or CSF. The representative DNA amplification method is the PCR assay technique. In this section, an overview of the principles of PCR assay techniques is presented, mainly with regard to their recent advancement and evolution.

4.1. The Basic Principle of PCR Assay. A schema indicating the basic principle of the PCR assay is shown in Figure 1(a).

Fundamentally, the PCR assay technique depends on “thermal cycling,” consisting of cycles with repeated heating and cooling for the reactions of DNA denaturation and enzymatic replication of DNA. Short DNA fragments called “primers” containing sequences complementary to the target region along with a DNA polymerase are key components to enable sequence-specific DNA amplification. The thermal cycling procedure involves a first step for physical separation of the two strands of DNA double helix at a high temperature (94–98°C for 20–30 seconds); this is called the DNA denaturation step. At a lower temperature (50–65°C for 20–40 seconds), the primers that are complementary to the target DNA region anneal to each separated single-stranded DNA as the template; this is called the annealing step. The specificity of PCR mainly results from both the primer sequence setting and the annealing temperature setting depending on the length of primers. The DNA polymerase binds to the primer-template hybrid and begins DNA synthesis. The DNA polymerase enzymatically assembles and synthesizes a new DNA strand complementary to the DNA template in the 5′ to 3′ direction; this is called the extension/elongation step. In general, almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. As a result, it is possible to repeat serially the thermal cycling procedure, consisting of alternate heating and cooling steps. As the thermal cycling procedure progresses, the synthesized DNA fragment is itself used as a template for replication, setting in motion a “chain reaction” in which the DNA template is exponentially amplified.

Through the use of agarose gel electrophoresis, the amplified DNA fragments can be separated by their lengths (molecular weights). The agarose gel is then treated with a solution containing ethidium bromide (EtBr), which is the most commonly used dye for visualizing DNA bands in agarose gel electrophoresis. Because EtBr fluoresces under UV light when intercalated into the major groove of DNA, through EtBr treatment, the amplified target DNA fragment can be visualized and detected as a distinctive band.

4.2. The Principle of Nested PCR Assay. A schema indicating the principle of nested PCR assay is shown in Figure 1(b).

Nested PCR assay is a modified version of the PCR technique intended to increase the amplification efficiency markedly and to reduce the level of nonspecific PCR products due to the amplification of untargeted primer binding sites. Although the specificity of the standard single-step PCR depends on the primers’ complementarity to the target DNA sequence, a commonly occurring problem is that primers bind to other similar regions of the DNA, giving untargeted PCR products such as primer dimers, hairpins, and alternative primer target sequences. Nested PCR assay requires two sets of primer pairs, used in two successive steps of the PCR procedure. In particular, a second set of primer pairs is prepared to amplify a secondary target within the first-step PCR product; this is the source of the term “nested”. The first set of primers amplifies a fragment similarly to the standard single-step PCR. However, the second set of primers binds inside the first-step PCR product to allow amplification of the second-step PCR product, which is shorter than the first one. The advantage of the nested PCR assay is that, if an untargeted or nonspecific PCR product is amplified, the probability is quite small that the region would be amplified in the second-step PCR by the second set of primers. Thus, nested PCR assay is an excellent technique for obtaining a sufficient amount of target DNA through a two-step amplification procedure, and for prominently improving the specificity to the target sequence by reducing the amplification of nonspecific products.

4.3. The Principle of Real-Time Quantitative PCR Assay. Real-time PCR assay is a variation of the PCR technique intended to amplify and simultaneously quantify a targeted DNA molecule; it enables both detection and quantification. Although the basic procedure of real-time PCR follows the general principle of classical PCR, its key feature is that the amplified DNA fragment is detected as the reaction progresses in “real time.” This is a novel and revolutionary approach compared with conventional standard PCR, where the PCR product is detected at the end of the reaction procedure. Two common methods for detection of products in real-time PCR are as follows: (1) nonspecific fluorescent dyes such as SYBR Green that intercalate with any double-stranded DNA and (2) sequence-specific oligonucleotide probes such as TaqMan probe that are labeled with a fluorescent reporter dye, which permits detection only after hybridization of the probe with its complementary target sequence. The former (SYBR Green) will bind to all double-stranded DNA PCR products including nonspecific PCR products (such as primer dimer). This is a potential disadvantage as it could obstruct accurate quantification of the intended target DNA fragment.

In contrast, fluorescent reporter probes such as TaqMan probe detect only the DNA fragment containing the complementary probe sequence; therefore, use of such reporter probes significantly increases the specificity and enables accurate quantification, even in the presence of nonspecific amplified fragments. A schema indicating the principle of real-time quantitative PCR assay based on a fluorescent reporter probe (TaqMan PCR) is shown in Figure 1(c). The sequence-specific oligonucleotide probe is labeled with a fluorescent reporter dye, such as FAM or VIC, at the 5′-
end and conjugated with a quencher dye, such as TAMRA, at the 3’ end. The close proximity between a fluorescent reporter dye and a quencher dye prevents emission of its fluorescence. As the reaction is initiated, both probe and primers anneal to the target DNA sequence during the annealing step of PCR. As the PCR procedure progresses, breakdown of the probe by the 5’ to 3’ exonuclease activity of Taq polymerase separates the reporter from the quencher and thus allows unquenched emission of fluorescence of the reporter dye, which can be detected after excitation with a laser. The strength of fluorescence increases exponentially because fluorescent reporter dyes separate from quenchers in a manner corresponding to the progress of PCR cycles, and its geometric increase is used to determine the threshold cycle (Ct) value in order to calculate the amplification rate in each reaction. As a result, the primary amount of DNA can be quantified accurately.

5. Commercially Available NAA Methods for \textit{M. Tb} \[^{16}\]

Currently, two commercially available NAA methods for the direct detection of \textit{M. Tb} complex have been approved by the United States Food and Drug Administration (FDA), as follows: Roche Amplicor \textit{Mycobacterium tuberculosis} Test (Roche Diagnostic Systems, Inc., Indianapolis, IN, USA) and Gene-Probe Amplified \textit{Mycobacterium tuberculosis} Direct (MTD) Test (Gene-Probe, Inc., San Diego, CA, USA) \[^{2, 6–12}\]. Both tests use the 16S ribosomal (r) RNA gene of \textit{M. Tb} (GenBank accession no. NC_000962.2 (147184–1473382)) as the target sequence for amplification \[^{2, 6–12}\]. The 16S rRNA gene represents a stable property of microorganisms and is widely used as the target for identifying mycobacterial species \[^{2, 6–12}\].

Roche Amplicor Test involves the conventional standard PCR-based method. In this test kit, the DNA fragment is amplified and detected by the primer pair and probe that are specific for the 16S rRNA gene of \textit{M. Tb} \[^{2, 6, 8, 10}\]. In addition, Roche Cobas TaqMan MTB Test is the improved successor to the Amplicor Test and adopts a real-time (TaqMan) PCR assay technique. Meanwhile, Gen-Probe MTD Test is the isothermal amplification method for RNA \[^{2, 7, 9–12}\]. In this test kit, the 16S rRNA of \textit{M. Tb} is directly amplified by the coupling of reverse transcriptase and RNA polymerase under a constant temperature (43°C), and detected by hybridization using specific oligo-RNA
probe [2, 7, 9–12]. At present, Amplicor is approved by the FDA for testing of AFB smear-positive respiratory specimens only [2, 6–12]. Meanwhile, MTD is approved for testing of respiratory specimens, regardless of the result of smear for AFB [2, 6–12]. Several studies have reported excellent results for both tests (sensitivity and specificity levels of more than 95%) in AFB smear-positive respiratory specimens, but reduced sensitivity (60 to 85%) when applied for AFB smear-negative respiratory specimens [2, 6–12]. Neither test is approved by the FDA for testing of CSF specimens [2, 6–12].

6. Clinical Application of PCR Assay Technique for the Diagnosis of CNS Tuberculosis

Table 1 summarizes the performance of PCR-based assays for the diagnosis of CNS tuberculosis [2, 6–42]. The challenges of applying NAA assay techniques to the rapid diagnosis of M.Tb in the CSF specimens stem largely from the small number of bacilli typically present in TBM and the presence of amplification inhibitors in the CSF [2–42]. In actual clinical practice, the sensitivity and specificity of PCR-based assay methods are the most serious issues in the diagnosis of CNS tuberculosis. In order to improve both the sensitivity and the specificity of PCR assay, the efficient extraction and purification of DNA from a small number of M.Tb bacilli in the CSF specimens and the setting of primers to amplify M.Tb DNA as the template specifically and efficiently are the most important factors [2–42]. Therefore, many researchers have worked intensively on these issues [2–42]. In the 1990s, a number of improved extraction and purification methods of M.Tb DNA from CSF specimens were reported by Shankar et al. [14], Kaneko et al. [13], and Lin et al. [20]. According to these studies, the CSF specimen was treated with cytolysis buffer containing proteinase-K and sodium dodecyl sulfate (SDS) as a surface-active agent, and then M.Tb DNA was extracted and purified using phenol-chloroform and ethanol precipitation from the treated CSF specimen [13, 14, 20]. In order to extract a small amount of M.Tb DNA from a CSF specimen more efficiently, the authors used a high-molecular-weight carrier, Ethachinmate (Nippon Gene, Tokyo, Japan), as a coprecipitating agent for the nucleotides together with the previously reported phenol-chloroform extraction and ethanol precipitation [3, 4, 32, 33, 38, 40, 41]. However, in recent studies, the conventional phenol-chloroform extraction and ethanol precipitation have tended to be regarded as inadequate for routine use in clinical examinations because of their laborious and time-consuming procedures [25–31, 34–37]. Generally, commercially available column extraction kits, such as the QIAamp Blood Kit and QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA), and the Cobas Amplicor respiratory specimen preparation kit (Roche Diagnostic Systems, Inc., Indianapolis, IN, USA), have been widely used for DNA extraction from various samples in previous studies [6–12, 25–31, 34–37]. However, in the current study, it was impossible to extract M.Tb DNA from CSF specimens sufficiently using commercial column extraction kits; therefore, these popular kits may be inadequate for extracting a small amount of M.Tb DNA from CSF specimens [3, 4, 32, 33, 38, 40, 41].

Currently, the four major M.Tb DNA-specific sequences, including the regions of IS6110 insertion sequence (Rv3475: GenBank accession no. NC_000962.2 (3891051–3892091)), 65-kDa heat shock protein antigen (Rv0251c: NC_000962.2 (302173–302652)), 16S rRNA gene and MPT64 (NC_000962.2 (2223343–2224029)) are evaluated by NAA assays (Table 1) [6–42]. Of these four sequence regions, the IS6110 insertion sequence, which is a repetitive element exclusively found in the genome of M.Tb complex, has been most widely used as the target sequence with superior amplification efficiency in many studies (Table 1) [15, 16, 18, 19, 21, 22, 24, 25, 28, 34, 36, 37, 39]. In these previous studies, PCR assays targeting the IS6110 insertion sequence revealed relatively good results (an overall sensitivity of 70–98% and specificity of 80–100%) for TBM diagnosis (Table 1). In addition, next to the IS6110 insertion sequence, the 16S rRNA gene has been frequently used for NAA assays, and it is the target sequence of two commercially available M.Tb detection methods, namely, the Roche Amplicor Test and the Gen-Probe MTD Test, which have been approved by the FDA for testing of respiratory specimens, as described above [2, 6–12]. At present, no commercially available NAA assay methods have been approved for testing of CSF, but several studies have evaluated their performance in TBM cases (Table 1) [2, 6–12]. A recent meta-analysis concerning the accuracy of commercially available NAA assay methods for TBM diagnosis revealed an overall sensitivity of 56% and specificity of 98% [10]. On the basis of these results, the commercially available NAA assay methods are evaluated as follows: they may play a role in confirming TBM, but because of low sensitivity, they are not ideal for ruling out TBM [2, 10]. As the major reason for the insensitive performance of these two commercially available NAA assay methods for TBM diagnosis, it is considered that, since they have been approved for testing of respiratory specimens containing relatively large numbers of M.Tb bacilli, they are inadequate for detecting a small amount of M.Tb DNA from CSF specimens. Recently, in several studies, Gen-Probe MTD Tests modified to improve the performance for detecting M.Tb DNA in CSF specimens have been reported [7, 9, 11, 12]. However, the use of these modified techniques was limited to a single laboratory, and they have not been widely used [7, 9, 11, 12]. Meanwhile, MPT64 is the gene sequence encoding the MPB64 protein that is specific for M.Tb complex and exists at only one site on the M.Tb genome. Therefore MPT64 is regarded as the most specific sequence for PCR assays and has been used as the target sequence for PCR in many studies (Table 1) [3, 4, 13, 14, 16, 17, 20, 23, 26, 27, 32, 33, 38, 40, 41]. Lee et al. [16] compared three sequence regions (IS6110, 65 kDa antigen, and MPT64) and reported that MPT64 was the most specific and sensitive sequence for the amplification of M.Tb DNA by PCR.

Beyond the commercially available NAA methods, the application of PCR-based methods to amplify M.Tb DNA has received a lot of attention clinically (Table 1) [13–42]. As described above, in order to improve the performance of
### Table 1: Performance of PCR-based assays for the diagnosing TBM.

<table>
<thead>
<tr>
<th>Author</th>
<th>Reported year</th>
<th>Assay technique</th>
<th>Specimens and cases</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercially available NAA assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonington et al.</td>
<td>1998</td>
<td>Roche amplicor PCR</td>
<td>83 CSF/69 patients (40 TBM, 29 non-TBM): South Africa</td>
<td>60</td>
<td>100</td>
<td>[6]</td>
</tr>
<tr>
<td>Lang et al.</td>
<td>1998</td>
<td>Modified Gen-Probe MTD</td>
<td>84 CSF and children (24 TBM, 60 non-TBM): Dominica</td>
<td>83</td>
<td>100</td>
<td>[7]</td>
</tr>
<tr>
<td>Bonington et al.</td>
<td>2000</td>
<td>Roche Cobas Amplicor PCR</td>
<td>83 CSF/69 patients (40 TBM, 29 non-TBM): South Africa</td>
<td>17.5</td>
<td>100</td>
<td>[8]</td>
</tr>
<tr>
<td>Chedore and Jamieson</td>
<td>2002</td>
<td>Gen-Probe MTD</td>
<td>311 CSF: Canada</td>
<td>193.8</td>
<td>99.3</td>
<td>[9]</td>
</tr>
<tr>
<td>Pai et al.</td>
<td>2003</td>
<td>Review and Meta-Analysis</td>
<td>14 studies with commercial NAA assays</td>
<td>56</td>
<td>98</td>
<td>[10]</td>
</tr>
<tr>
<td>Cloud et al.</td>
<td>2004</td>
<td>Modified Gen-Probe MTD</td>
<td>27 CSF specimens spiked with M. tuberculosis</td>
<td>17–100</td>
<td>100</td>
<td>[12]</td>
</tr>
<tr>
<td>Other PCR-based assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaneko et al.</td>
<td>1990</td>
<td>MPT64 single PCR</td>
<td>26 CSF and patients (6 TBM, 20 non-TBM): Japan</td>
<td>83.3</td>
<td>100</td>
<td>[13]</td>
</tr>
<tr>
<td>Shanker et al.</td>
<td>1991</td>
<td>MFT64 single PCR</td>
<td>85 CSF and patients (34 TBM, 51 non-TBM): India</td>
<td>65</td>
<td>88</td>
<td>[14]</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>1994</td>
<td>IS6110/65kDa antigen/MFT64 PCR</td>
<td>27 CSF and patients (6 TBM, 21 non-TBM): Singapore</td>
<td>100/83/83</td>
<td>38/67/90</td>
<td>[16]</td>
</tr>
<tr>
<td>Liu et al.</td>
<td>1994</td>
<td>MFT64 nested PCR</td>
<td>100 CSF and patients (21 TBM, 79 non-TBM): Singapore</td>
<td>90</td>
<td>100</td>
<td>[17]</td>
</tr>
<tr>
<td>Folgueira et al.</td>
<td>1994</td>
<td>IS6110 single PCR</td>
<td>25 AIDS patients (11 TBM, 14 non-TBM): Spain</td>
<td>82</td>
<td>100</td>
<td>[18]</td>
</tr>
<tr>
<td>Scarpellini et al.</td>
<td>1995</td>
<td>IS6110 nested PCR</td>
<td>68 CSF/36 AIDS patients (12 TBM, 24 non-TBM): Italy</td>
<td>100</td>
<td>100</td>
<td>[19]</td>
</tr>
<tr>
<td>Lin et al.</td>
<td>1995</td>
<td>MFT64 single PCR</td>
<td>47 CSF/45 patients (18 TBM, 27 non-TBM): Taiwan</td>
<td>70</td>
<td>100</td>
<td>[20]</td>
</tr>
<tr>
<td>Kox et al.</td>
<td>1995</td>
<td>IS6110 single PCR</td>
<td>42 patients (24 TBM, 18 non-TBM): The Netherlands</td>
<td>48</td>
<td>100</td>
<td>[21]</td>
</tr>
<tr>
<td>Nguyen, et al.</td>
<td>1996</td>
<td>IS6110 single PCR</td>
<td>136 TBM patients: Vietnam</td>
<td>32</td>
<td>100</td>
<td>[22]</td>
</tr>
<tr>
<td>Seth et al.</td>
<td>1996</td>
<td>MFT64 single PCR</td>
<td>89 CSF and patients (40 TBM, 49 non-TBM): India</td>
<td>85</td>
<td>94</td>
<td>[23]</td>
</tr>
<tr>
<td>Wei et al.</td>
<td>1999</td>
<td>IS6110/65kDa antigen/MFT64 multiplex nested PCR</td>
<td>11 CSF and patients (5 TBM, 6 non-TBM): China</td>
<td>60</td>
<td>66</td>
<td>[24]</td>
</tr>
<tr>
<td>Caws et al.</td>
<td>2000</td>
<td>IS6110 nested PCR</td>
<td>131 TBM patients: United Kingdom</td>
<td>175</td>
<td>94</td>
<td>[25]</td>
</tr>
<tr>
<td>Martins et al.</td>
<td>2000</td>
<td>MFT64 nested PCR</td>
<td>73 specimens (30 PF, 26 PB, 17 CSF): Brazil</td>
<td>170</td>
<td>88</td>
<td>[26]</td>
</tr>
<tr>
<td>Brienza et al.</td>
<td>2001</td>
<td>MPT64 nested PCR</td>
<td>91 patients (41 TBM, 50 non-TBM): Brazil</td>
<td>53</td>
<td>100</td>
<td>[27]</td>
</tr>
<tr>
<td>Narayanan et al.</td>
<td>2001</td>
<td>IS6110/TRC4 single PCR</td>
<td>96 CSF and patients (67 TBM, 29 non-TBM): India</td>
<td>80.5/91</td>
<td>79/76</td>
<td>[28]</td>
</tr>
<tr>
<td>Desai et al.</td>
<td>2002</td>
<td>pKSIO single PCR</td>
<td>120 CSF and patients (105 TBM, 15 non-TBM): India</td>
<td>31</td>
<td>100</td>
<td>[29]</td>
</tr>
<tr>
<td>Rafi and Naghidy</td>
<td>2003</td>
<td>Single PCR (target not available)</td>
<td>36 CSF and patients (29 TBM, 6 non-TBM): Iran</td>
<td>86.2</td>
<td>100</td>
<td>[30]</td>
</tr>
<tr>
<td>Kulkarni et al.</td>
<td>2005</td>
<td>38 kDa protein single PCR</td>
<td>60 CSF and patients (30 TBM, 30 non-TBM): India</td>
<td>90</td>
<td>100</td>
<td>[31]</td>
</tr>
<tr>
<td>Takahashi et al.</td>
<td>2005</td>
<td>MPT64 nested PCR</td>
<td>29 CSF and patients (9 TBM, 20 non-TBM): Japan</td>
<td>100</td>
<td>100</td>
<td>[32]</td>
</tr>
<tr>
<td>Takahashi and Nakayama</td>
<td>2006</td>
<td>MPT64 QNRT-PCR</td>
<td>29 CSF and patients (9 TBM, 20 non-TBM): Japan</td>
<td>100</td>
<td>100</td>
<td>[33]</td>
</tr>
<tr>
<td>Quan et al.</td>
<td>2006</td>
<td>IS61 10 single PCR</td>
<td>74 CSF and patients (25 TBM, 49 non-TBM): China</td>
<td>75</td>
<td>93.7</td>
<td>[34]</td>
</tr>
<tr>
<td>Desai et al.</td>
<td>2006</td>
<td>Single PCR (target not available)</td>
<td>57 CSF and patients (30 TBM, 27 non-TBM): India</td>
<td>66.7</td>
<td>100</td>
<td>[35]</td>
</tr>
</tbody>
</table>
Various ideas and modifications have already been tried [2, 6–42]. Recently, as a revolutionary assay technique that conveys drastically increased sensitivity and specificity compared with the conventional standard PCR assay, nested PCR assay has been innovated for the diagnosis of CNS tuberculosis [3, 4, 17, 19, 24–27, 32]. Liu et al. [17] reported that nested PCR assay targeting MPT64 was performed for CSF specimens collected from 21 patients with clinically suspected TBM and enabled diagnosis of TBM with a sensitivity of 90% and a specificity of 100% within 24 hours. In addition, they reported that the nested PCR assay had approximately 1000 times higher sensitivity than the conventional single-step PCR assay [17]. The authors performed the nested PCR assay targeting MPT64 for the CSF specimens collected from 9 patients with clinically highly suspected TBM [32]. In our study, both the sensitivity and the specificity of nested PCR assay were 100%, but the sensitivity of the conventional single-step PCR assay and culture for M. Tb was only 22.2% [32]. Moreover, the minimum detection sensitivity of our nested PCR assay technique was examined. This technique enabled detection at a level as low as 1–10 copies/2 μL of purified M. Tb DNA and had 1000 to 10,000 times higher sensitivity than the conventional single-step PCR assay [32]. Concerning the relationship between the PCR assay results and the responses for ATT, Lin et al. [20] examined this relationship by conventional single-step PCR assay and Scarpellini et al. [19] examined it by nested PCR assay. In particular, Scarpellini et al. [19] performed diachronic study by nested PCR assay targeting IS6110 for serial CSF specimens collected from 7 TBM patients during the clinical treatment course. In their diachronic study, the nested PCR assay results were converted from positive to negative in 4 TBM patients whose clinical conditions recovered during ATT [19]. In contrast, the nested PCR assay results remained positive throughout the clinical course in 3 patients who demonstrated no ATT response and died [19]. Therefore, Scarpellini et al. concluded that the nested PCR assay was useful for assessing the clinical treatment course of TBM [19]. Similarly, in our study, 11 out of 27 serial CSF specimens that were available from the 7 patients with highly suspected TBM and collected during the clinical treatment course of ATT revealed positive results (40.7%) of the nested PCR assay targeting MPT64 [32]. Moreover, our nested PCR assay results were converted from positive to negative in the 6 patients whose clinical conditions recovered during the ATT [32]. In contrast, the conventional methods including single-step PCR assay and culture for M. Tb all revealed negative results for a series of 27 CSF specimens during the clinical treatment course of 7 patients [32]. The nested PCR assay is a useful assay technique with superior sensitivity and specificity. Because of the demonstration of the capacity of the nested PCR assay in the diagnosis of difficult cases in which other conventional assay methods cannot detect M. Tb, the authors speculated that, if this assay technique was widely and appropriately used within clinical practice, it would be a powerful tool for the rapid and accurate diagnosis of CNS tuberculosis.

Currently, although the wide use of nested PCR assay in clinical practice is expected, regrettably, it has rarely been performed for TBM diagnosis [3, 4, 17, 19, 24–27, 32]. The main argument against the use of nested PCR assay is that, because of its complicated, laborious, and time-consuming procedures, it is an inadequate assay technique for routine use in clinical examination [3, 4, 17, 19, 24–27, 32]. In addition, owing to its markedly increased sensitivity and the requirement for an additional amplification step, in general, it is considered that cross-contamination can easily occur through the nested PCR assay procedure [3, 4, 17, 19, 24–27, 32]. However, the possibility of cross-contamination can be minimized by good laboratory practice. Certainly, the nested PCR assay may be inadequate for routine use in clinical examinations dealing with many sample specimens such as screening examinations. However, even if any other “simple” assay methods are used for TBM diagnosis, a negative result cannot exclude the possibility of M. Tb infection, and clinical judgment in TBM diagnosis remains a serious problem with the requirement of an appropriate clinical examination [2]. In actual clinical practice, the diagnosis of CNS tuberculosis requires not a screening examination but rather a definitive examination. Therefore, the nested PCR assay should become a prominent and useful assay technique

<table>
<thead>
<tr>
<th>Author</th>
<th>Reported year</th>
<th>Assay technique</th>
<th>Specimens and cases</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rafi et al.</td>
<td>2007</td>
<td>IS6110 single PCR, MPT64/65kDa antigen nested PCR</td>
<td>176 CSF and patients (75 TBM, 101 non-TBM): India</td>
<td>98/91/51</td>
<td>100/91/92</td>
<td>[36]</td>
</tr>
<tr>
<td>Rafi and Naghilys</td>
<td>2007</td>
<td>IS6110 uniplex (single) PCR</td>
<td>945 CSF and patients (677 TBM, 268 non-TBM): India</td>
<td>76.4</td>
<td>89.2</td>
<td>[37]</td>
</tr>
<tr>
<td>Takahashi et al.</td>
<td>2007</td>
<td>MPT64 QNRT-PCR</td>
<td>63 CSF/28 patients (8 TBM, 20 non-TBM): Japan</td>
<td>55.8</td>
<td>100</td>
<td>[38]</td>
</tr>
<tr>
<td>Deshpande et al.</td>
<td>2007</td>
<td>IS6110 Single PCR</td>
<td>80 CSF and patients (51 TBM, 29 non-TBM): India</td>
<td>91.4</td>
<td>75.9</td>
<td>[39]</td>
</tr>
<tr>
<td>Takahashi et al.</td>
<td>2008</td>
<td>MPT64 WR-QNRT-PCR</td>
<td>96 CSF/53 patients (24 TBM, 29 non-TBM): Japan</td>
<td>95.8</td>
<td>100</td>
<td>[40, 41]</td>
</tr>
<tr>
<td>Haldar et al.</td>
<td>2009</td>
<td>devR qRT-PCR</td>
<td>167 CSF and patients (81 TBM, 86 non-TBM): India</td>
<td>87.6</td>
<td>92</td>
<td>[42]</td>
</tr>
</tbody>
</table>

if used on well-defined and appropriate clinical specimens collected from “highly suspected” TBM patients. Finally, a remaining challenge of using PCR-based assay methods in the diagnosis of CNS tuberculosis is the requirement for an appropriate laboratory infrastructure to perform these more sophisticated assay techniques; it is a fact that the infrastructure is often lacking in areas where TBM is highly endemic. This is a crucial issue that should be solved as soon as possible in the diagnosis of CNS tuberculosis.

7. Development of Novel Diagnostic Assay Technique Based on PCR for CNS Tuberculosis

Recently, the authors developed an internally controlled novel “wide-range (WR)” quantitative nested real-time (QNRT) PCR assay for M.Tb DNA, in order to rapidly diagnose CNS tuberculosis [32, 33, 38, 40, 41]. This technique combines the high sensitivity of nested PCR with the accurate quantification of real-time (TaqMan) PCR (Figure 2(a)) [40].

In WR-QNRT-PCR assay, two types of original plasmid, “wild” (W) and “new mutation” (NM) plasmids, were prepared for quantitative detection of M.Tb DNA [40, 41]. W-plasmid, which was inserted a 239-base-pair (bp) DNA fragment of the MPT64 gene into pCR 2.1 vector (Invitrogen Corp., San Diego, CA, USA), was constructed for use as standard template (Figure 2(b)) [40]. NM-plasmid was developed on the basis of the W-plasmid for use as a new internal control “calibrator” in WR-QNRT-PCR assay (Figure 2(b)) [40]. In NM-plasmid, a total of five regions, where two pairs of (outer and inner) forward and reverse primers and TaqMan probe annealed, were replaced with artificial random nucleotides (Figure 2(b)) [40]. The sequences of the artificial random nucleotides were set to have the same nucleotide composition as MPT64 of wild M.Tb [40, 41]. For use in WR-QNRT-PCR assay, four pairs of specific primers and two types of specific TaqMan probes were prepared. The sequences and positions of these primers and probes are shown in Table 2 and Figure 2(b) [40, 41]. The two pairs of forward and reverse primers (outer primer pair MF1 and WR1 for use at the first step and inner primer pair TqMn-MF2 and TqMn-MR2) and TaqMan probe (TqMn-M-FAM) were specific for the artificial random nucleotides in NM-plasmid for use as a new internal control “calibrator.” These primers and probes were set to have the same nucleotide composition but a different and random sequence (Table 2) [40, 41]. Therefore, the annealing efficiencies of these primers and probes to wild MPT64 or NM-plasmid as a template can be regarded as the same.

WR-QNRT-PCR assay consists of two consecutive PCR amplification steps, which are conventional PCR at the first step and nested real-time PCR at the second step.
**Figure 2:** Continued.
**Figure 2**: The principle of wide-range (WR) quantitative nested real-time (QNRT) PCR assay and its results. (a) A schema indicating the principle of WR-QNRT-PCR assay. (b) A schema of wild (W) and new mutation (NM) plasmids NM-plasmid was developed for use as a new internal control. (c) The specific standard curve to detect *Mycobacterium tuberculosis* (*M.Tb*) DNA or W-plasmid quantitatively. (d) The specific standard curve to detect the NM-plasmid as a new internal control quantitatively. (e) Amplification curves for 10^3 copies of NM-plasmids as a new internal control. (f) One-way ANOVA against Ct values for 10^3 copies of NM-plasmid. (g) The progress of *M.Tb* DNA copy numbers calculated by the WR-QNRT-PCR assay during clinical time course in 10 suspected tuberculous meningitis (TBM) patients (cases 3 and 8–16). Statistical comparison between the cases in which anti-tuberculosis treatment (ATT) was effective (cases 8–14 and 16) and those in which it was not effective (cases 3 and 15) was carried out by repeated-measure ANOVA. (h) Cranial MRI findings for cases 11 and 12 on admission. 1, 2: Cranial MRI findings for case 11. 1: FLAIR image (TR 9000/TE 110). High-intensity lesions of cerebral infarction (circle), which were probably caused by tuberculous vasculitis, are noted on both sides of the frontal lobe. 2: T1-WI (TR 500/TE 17). A cranial tuberculoma was noted in the right thalamus. 3, 4, 5: Cranial MRI findings (Gd T1-WI: TR 400/TE 15) for case 12. Multiple cranial tuberculomas with marked Gd enhancement (arrows) were noted (3: midbrain, 4: right cerebellum, 5: right temporal lobe). (i) Result of simple regression analysis between *M.Tb* DNA copy number (y axis) and clinical stage of TBM (x axis).

Statistically analyzed by the one-way ANOVA

NSD: $F = 1.086, P = 0.774$

Statistically analyzed by

the one-way ANOVA

$R^2 = 0.597, *P < 0.0001$

first step and real-time (TaqMan) PCR at the second step (Figure 2(a)) [33, 38, 40, 41]. In this assay, the entire procedure including extraction, amplification, and detection for both *M.Tb* DNA and NM-plasmid as a new internal control is performed simultaneously under the same assay conditions by using four pairs of primers and two probes that have equivalent annealing efficiency to the respective templates. Therefore, the initial copy number of *M.Tb* DNA in CSF samples can be calculated by the amplification ratio against the new internal control (NM plasmid) as a “calibrator,” using the following equation: $X : W = C : M : X = W \times C / M$. ($X$, the initial copy number of *M.Tb* DNA per 1 mL of CSF sample; $C$, the initial copy number of the new internal control (=“calibrator” 10^3 copies of NM-plasmid); $W$ and $M$, the copy numbers of *M.Tb* DNA and NM-plasmid, respectively, after passing through extraction and PCR amplification procedures.) [40, 41] In *M.Tb*, it is universally acceptable that a single copy of MPT64 gene...
represents one bacterial cell. Therefore, it can be considered that the copy numbers calculated by WR-QNRT-PCR assay correspond to the bacterial cell numbers of *M. Tb* in CSF samples.

For WR-QNRT-PCR assay, two specific standard curves for quantitative detection of *M. Tb* DNA and NM-plasmid as a new internal control are needed [40, 41]. The precision of the standard curve is the principal factor for quantitative detection in real-time (TaqMan) PCR assay [40, 41]. The two specific standard curves are shown in Figures 2(c) and 2(d) [40, 41]. In simple regression analysis, both of these two standard curves demonstrated a significant linear relationship ($R^2 > 0.99$) between the threshold cycle (Ct) values (y axis) and log of the starting copy numbers for each standard template (x axis). In both standard curves, no significant differences were found among the plots in repeated experiments ($n = 10$; $F = 1.007$, $P = 0.65$ and $F = 1.015$, $P = 0.53$) by two-way ANOVA. The PCR-efficiency (Eff) of real-time PCR can be calculated by the slope of the standard curve, in the following equation: $\text{PCR - Eff} = 10^{\frac{1}{\text{slope}}} - 1$. The PCR-Eff calculated by this equation based on the slopes (-3.33 or -3.28) of two standard curves was 99.7 or 101.8%, respectively [40, 41]. These results indicated that the efficiencies of amplification and detection for both *M. Tb* DNA and the new internal control were almost equivalent in the WR-QNRT-PCR assay.

When a value of $10^3$ copies of NM-plasmid was set as the optimal copy number of new internal control, the amplification curves of NM-plasmids revealed extremely uniform patterns in all starting copy numbers ($1–10^5$) of W-plasmids as a mimic of *M. Tb* DNA (Figure 2(e)) [40]. In addition, the Ct values for $10^3$ copies of the NM-plasmid also revealed statistically significant uniform variance between all starting copy numbers of W-plasmids ($F = 1.086$, $P = 0.774$) by one-way ANOVA (Figure 2(f)) [40]. These results indicated that there was no interference between *M. Tb* DNA and the new internal control in the entire PCR amplification procedures. Therefore, NM-plasmid could be regarded as appropriate for use as a new internal control "calibrator" in WR-QNRT-PSR assay. Owing to the development of NM-plasmid, the WR-QNRT-PCR assay enabled statistically significant stable and accurate quantitative detection of *M. Tb* DNA with a wide detection range ($1–10^5$ copies) [40, 41].

The authors examined the clinical usefulness of the WR-QNRT-PCR assay for rapid and accurate diagnosis and assessment of the clinical course of CNS tuberculosis [41]. Twenty-four patients with clinically suspected TBM and 29 non-TBM control patients were collected between 1998 and 2005 [41]. A total of 67 CSF samples were collected from these 24 patients. Of 67 CSF samples, 43 were available serially from the 10 patients (cases 3 and 8–16) who had followups of more than 2 weeks [41]. Table 3 summarizes the clinical features of the 24 suspected TBM patients upon admission (before ATT) [41]. All 24 patients met the (A) clinical criteria and (B) supporting evidence for TBM (shown in Table 3) and were classified as 8 "confirmed" cases (cases 1 to 8) (CSF culture positive for *M. Tb*) and 16 "highly probable" cases (cases 9 to 24) (meeting all the clinical criteria and with three positive results for supporting evidence, but having no bacterial isolation). In clinical applications, the WR-QNRT-PCR assay revealed sufficiently high sensitivity (95.8%) and specificity (100%) for 24 clinically suspected TBM patients [41]. The measured copy numbers of *M. Tb* DNA (per 1 mL of CSF) are shown in Table 3 [41]. In conditional logistic regression analysis, a copy number of *M. Tb* DNA (per 1 mL CSF) > 8000 was an independent risk factor for poor prognosis of TBM (=death) ($OR = 16.142$, 95%CI = 1.191–218.79, $P = 0.0365$) [41]. In the diachronic study, the copy numbers of *M. Tb* DNA demonstrated significant alterations during the clinical treatment course in 10 suspected TBM patients, in these 10 patients including 2 "confirmed" cases (cases 3 and 8) and 8 "highly probable" cases (cases 9–16) [41]. The classical cultures for *M. Tb* revealed positive results in only three out of 43 serial CSF samples collected during the clinical treatment course in cases 3 and 8. In contrast, the quantitative detection of *M. Tb* DNA was possible in 25 CSF samples (58.1%) in the WR-QNRT-PCR assay [41]. In addition, the copy numbers of *M. Tb* DNA decreased gradually to below the detection limit of the WR-QNRT-PCR assay in the 8 patients (cases 8–14 and 16) for whom ATT was effective and who demonstrated improvement in both clinical stages and routine CSF findings during clinical treatment course (Figure 2(g)) [41]. However, in cases 3 and 15, in whom ATT was not effective and who died due to aggravation of TBM, the copy numbers were continually high throughout the clinical course (Figure 2(g)) [41]. The trend in the alterations of *M. Tb* DNA copy numbers during clinical treatment course demonstrated a significant difference ($P = 0.0041$) between the ATT effective cases (cases 8–14 and 16) and the ATT noneffective cases (cases 3 and 8) by repeated-measure ANOVA (Figure 2(g)) [41]. In cases 11 and 12, initial cranial MRI on admission demonstrated multiple intracranial focal masses that were regarded as typical tuberculosis (Table 4 and Figure 2(h)) [38, 41]. In these two cases with tuberculosis, after conversion to negative results of WR-QNRT-PCR assay, transient positivity was once again revealed without any symptoms of meningitis during the course of ATT [38, 41]. In general, tuberculosis often occur along with TBM, but certainly may occur in the absence of TBM [2]. Tuberculous in these two cases disappeared at 5 and 3 months after the initiation of ATT during the follow-up MRI (Table 4) [38, 41]. Interestingly, WR-QNRT-PCR assay results became completely negative along with the disappearance of tuberculosis [38, 41]. The authors speculate that the transient detections of *M. Tb* DNA by WR-QNRT-PCR assays in these two cases during the ATT were correlated with tuberculous. A small amount of *M. Tb* might have survived within the tuberculous. The high sensitivity of WR-QNRT-PCR assay might have detected a little DNA of extinct *M. Tb* leaking into CSF along with the rupture of tuberculous by ATT. These findings suggest that the combination of molecular-based techniques and neuroradiographic techniques is a promising and powerful diagnostic tool for TBM and tuberculomas in actual clinical practice. To our knowledge, there have been no previous reports on cases such as these two cases; therefore, they may be regarded as clinically important. Moreover, in simple
Table 3: The summary of basal clinical features in 24 patients with suspected TBM.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Clinical stage</th>
<th>Basal CSF findings (before treatment)</th>
<th>Single PCR assay</th>
<th>Nested PCR assay</th>
<th>WR-QNRT-PCR assay (copies/mL CSF)</th>
<th>Cranial MRI findings</th>
<th>M.Tb outside CNS</th>
<th>Period up to initial sample collection</th>
<th>ATT response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73/M</td>
<td>III</td>
<td>288/299 13 23.4 + + + 28721</td>
<td>ME, HC, CVD, IFM</td>
<td>Sp, GA</td>
<td>About 3 weeks</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>76/M</td>
<td>III</td>
<td>165/569 46 12.3 + + + 10028</td>
<td>ME, CVD</td>
<td>Sp</td>
<td>2 days</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>28/M</td>
<td>III</td>
<td>605/434 25 16.3 + + + 22571</td>
<td>ME, HC, CVD, IFM</td>
<td>Sp</td>
<td>About 1 month</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>38/M</td>
<td>II</td>
<td>76/637 18 6.5 + + + 7161</td>
<td>HC, IFM</td>
<td>Sp, GA</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>53/F</td>
<td>III</td>
<td>344/354 38 10.3 + + + 4547</td>
<td>IFM</td>
<td>–</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72/F</td>
<td>III</td>
<td>247/329 57 18.4 + + + 6340</td>
<td>HC, IFM</td>
<td>–</td>
<td>7 days</td>
<td>Noneffective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>34/M</td>
<td>II</td>
<td>612/320 18 20.2 + + + 1243</td>
<td>–</td>
<td>–</td>
<td>Not available</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>42/M</td>
<td>II</td>
<td>418/456 36 22.6 + + + 10532</td>
<td>ME, IFM</td>
<td>Sp</td>
<td>About 2 weeks</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>35/F</td>
<td>II</td>
<td>208/300 13 16.3 + + + 7892</td>
<td>ME, HC, CVD, IFM</td>
<td>–</td>
<td>7 days</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>65/F</td>
<td>I</td>
<td>107/70 48 7.8 – – – + 1904</td>
<td>–</td>
<td>–</td>
<td>3 days</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>52/M</td>
<td>II</td>
<td>18/135 54 8.6 – – – + 5858</td>
<td>ME, HC, CVD, IFM</td>
<td>Sp, GA</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>24/F</td>
<td>I</td>
<td>30/25 30 4.4 – – – + 5436</td>
<td>IFM</td>
<td>–</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>44/F</td>
<td>III</td>
<td>60/70 52 N.D. – – – + 9600</td>
<td>CVD</td>
<td>–</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>59/F</td>
<td>II</td>
<td>40/359 78 3.7 – – – + 5112</td>
<td>HC</td>
<td>–</td>
<td>About 1 month</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>44/M</td>
<td>III</td>
<td>117/87 48 3.9 – – – + 8400</td>
<td>ME</td>
<td>Sp, GA</td>
<td>About 3 weeks</td>
<td>Noneffective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>40/M</td>
<td>III</td>
<td>800/188 66 12 – – – + 7050</td>
<td>CVD</td>
<td>–</td>
<td>1 day</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>30/F</td>
<td>III</td>
<td>720/211 50 9.7 – – – + 5596</td>
<td>IFM</td>
<td>–</td>
<td>5 days</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>20/F</td>
<td>II</td>
<td>442/164 46 17.6 – – – – Not detected</td>
<td>IFM</td>
<td>GA</td>
<td>Not available</td>
<td>Effective</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Continued.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Clinical stage</th>
<th>Basal CSF findings (before treatment)</th>
<th>WR-QNRT-PCR assay (copies/mL CSF)</th>
<th>Cranial MRI findings</th>
<th>Period up to initial sample collection</th>
<th>ATT response</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>63/M</td>
<td>III</td>
<td>75 84 47 15.9 - - - -</td>
<td>76 ME</td>
<td>-</td>
<td>Not available</td>
<td>Effective</td>
</tr>
<tr>
<td>20</td>
<td>63/F</td>
<td>II</td>
<td>34 294 30 12.7 - - - +</td>
<td>188 HC</td>
<td>-</td>
<td>Not available</td>
<td>Effective</td>
</tr>
<tr>
<td>21</td>
<td>53/M</td>
<td>III</td>
<td>76 81 82 16.9 - - - +</td>
<td>2592 ME, IFM</td>
<td>-</td>
<td>1 day</td>
<td>Effective</td>
</tr>
<tr>
<td>22</td>
<td>51/M</td>
<td>III</td>
<td>227 155 34 12.7 - - - +</td>
<td>636 ME, CVD, IFM</td>
<td>-</td>
<td>1 day</td>
<td>Effective</td>
</tr>
<tr>
<td>23</td>
<td>66/M</td>
<td>III</td>
<td>129 120 58 4.7 - - - - +</td>
<td>1600 ME</td>
<td>-</td>
<td>4 day</td>
<td>Effective</td>
</tr>
<tr>
<td>24</td>
<td>2/F</td>
<td>II</td>
<td>193 119 30 8.3 - - - - +</td>
<td>1444 ME, HC</td>
<td>-</td>
<td>Not available</td>
<td>Effective</td>
</tr>
</tbody>
</table>

(a) According to the clinical stages defined by the British Medical Research Council: stage 0: no definite neurologic symptoms, stage I: slight signs of meningal irritation with slight clouding of consciousness, stage II: moderate signs of meningal irritation with moderate disturbance of consciousness and neurologic defects, stage III: severe disturbance of consciousness and neurologic defects.

Table 4: The detail of clinical treatment course of two patients (cases 11 and 12) who had tuberculomas.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serial clinical data</th>
<th>Time course</th>
<th>Months</th>
<th>ATT response</th>
<th>** Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (upon admission)</td>
<td>1 2 3 4 5 6 7 8</td>
<td>3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Before treatment</td>
<td>During treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Clinical stage</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Cells (µL)</td>
<td>18</td>
<td>23</td>
<td>25</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>135</td>
<td>62</td>
<td>46</td>
<td>69</td>
<td>42</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>54</td>
<td>71</td>
<td>78</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>M. Tb culture</td>
<td>−−−−−−−</td>
<td>Single PCR assay</td>
<td>−−−−−−−</td>
<td>Nested PCR assay</td>
<td>+</td>
</tr>
<tr>
<td>$M. Tb$ DNA copy number [WR-QNRT-PCR assay]</td>
<td>5860</td>
<td>8268</td>
<td>3624</td>
<td>1644</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cranial MRI findings</td>
<td>MF, CVD, IFM</td>
<td>CVD, IFM</td>
<td>CVD, IFM</td>
<td>CVD</td>
<td>N.D.</td>
</tr>
<tr>
<td>Treatment</td>
<td>INH (mg/day)</td>
<td>500</td>
<td>RFP (mg/day)</td>
<td>450</td>
<td>PZA (g/day)</td>
</tr>
<tr>
<td>Case 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Clinical stage</td>
<td>I</td>
<td>I</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cells (µL)</td>
<td>30</td>
<td>7</td>
<td>9</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>25</td>
<td>16</td>
<td>23</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>30</td>
<td>38</td>
<td>40</td>
<td>32</td>
<td>34</td>
</tr>
<tr>
<td>M. Tb culture</td>
<td>−−−−−−−</td>
<td>Single PCR assay</td>
<td>−−−−−−−</td>
<td>Nested PCR assay</td>
<td>+</td>
</tr>
<tr>
<td>$M. Tb$ DNA copy number [WR-QNRT-PCR assay]</td>
<td>5436</td>
<td>2292</td>
<td>N.D.</td>
<td>14720</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cranial MRI findings</td>
<td>MF, IFM</td>
<td>ME, IFM</td>
<td>IFM</td>
<td>CVD</td>
<td>N.D.</td>
</tr>
<tr>
<td>Treatment</td>
<td>INH (mg/day)</td>
<td>500</td>
<td>RFP (mg/day)</td>
<td>450</td>
<td>PZA (g/day)</td>
</tr>
</tbody>
</table>


* According to the clinical stages defined by the British Medical Research Council: stage 0: no define neurologic symptoms, stage 1: slight signs of meningeal irritation with slight clouding of consciousness, stage II: moderate signs of meningeal irritation with moderate disturbance of consciousness and neurologic defects, stage III: severe disturbance of consciousness and neurologic defects.

** Outcome classified as recovery with minor or no neurologic impairment, severe neurologic impairment, and death.
regression analysis, significant correlation ($R^2 = 0.597$, $P < 0.0001$) was demonstrated between $M.\text{Tb}$ DNA copy number and clinical stage of TBM (Figure 2(i)) [41]. These diachronic study results indicate that quantitative analysis by WR-QNRT-PCR assay is very useful for assessing the clinical course of TBM and ATT response [41]. To our knowledge, there have been no previous studies that serially assessed the quantity of DNA or bacterial cell numbers of $M.\text{Tb}$ in CSF samples throughout the clinical course of CNS tuberculosis [41]. In actual clinical application, the WR-QNRT-PCR assay demonstrated significant accuracy and reliability for quantitative detection of $M.\text{Tb}$ DNA in CSF samples owing to the development of NM-plasmid used as a new internal control. Therefore, this novel assay technique could be regarded as a useful and advanced method for rapidly and accurately diagnosing CNS tuberculosis [40, 41].

However, this novel assay technique is not widely used in routine clinical examination. It may be that the two consecutive amplification steps of WR-QNRT-PCR assay are regarded as a complicated and laborious procedure. Therefore, the authors are developing a further novel and “simpler and low-cost” assay technique with quantification and high-sensitivity almost equivalent to those with the WR-QNRT-PCR assay by one step of standard real-time PCR assay. This developing assay technique is a combination of the whole genome amplification (WGA) method with the real-time (TaqMan) PCR assay technique. The whole genome amplification (WGA) method provides a possibility of amplifying a small amount of high-quality DNA and there are several such techniques using commercially available kits at a reasonable price [50–52]. In particular, in these kits for the WGA method, a kit based on the multiple displacement amplification (MDA) technique has been found in many studies to provide the most balanced genome amplification [50–52]. The MDA technique is a superior method in which genomic DNA is continuously amplified by using Phi29 DNA polymerase of bacteriophage origin and random hexamer primers [50–52]. In this case, the authors used the GenomiPhi V2 kit (GE Healthcare Life Sciences, Uppsala, Sweden) based on the MDA technique. Because a small amount of $M.\text{Tb}$ DNA extracted and purified from a CSF specimen for use as a template can be amplified directly using the GenomiPhi V2 kit, the first-step PCR in the WR-QNRT-PCR assay can be omitted. This assay technique that is currently being developed can markedly simplify the procedure of WR-QNRT-PCR assay by innovation of the WGA method. Therefore, although it is yet to be reported, its wider use as a beneficial approach for clinical examination is expected in actual clinical practice.

As described above, CNS tuberculosis including TBM is the severest form of $M.\text{Tb}$ infection, causing death and serious sequelae [1–5]. In addition, owing to an increasing number of immunocompromised hosts caused by the prevalence of AIDS, increasing numbers of older people, and the wider use of immunosuppressive agents such as corticosteroid, CNS tuberculosis remains a serious clinical and social problem [1–5, 43–46]. In particular, in the underdeveloped and developing countries in Asia and Africa, in which there are many exacerbating social problems such as poverty, overcrowding, and malnutrition, and so forth, the epidemic of $M.\text{Tb}$ infection including TBM is regarded as a serious social and demographic crisis [1, 2, 43–46]. The authors consider that the novel assay techniques that we have developed are needed and should be used in these places. The wider use of our novel assay techniques will lead to an increase of the number of definitively diagnosed cases within the early stage of TBM and the improvement of treatment results for TBM; therefore, they should make a significant medical and social contribution in these countries. It is hoped that our efforts to develop novel and more rapid, accurate, highly sensitive, quantitative, simple, and low-cost assay techniques for the diagnosis of CNS tuberculosis will help to improve the level of medical care globally, particularly in underdeveloped or developing countries.

8. Conclusion

Recently, instead of the conventional “gold standard” based on bacteriological techniques, various approaches have been attempted for rapid and accurate diagnosis of CNS tuberculosis with high sensitivity. In this paper, an overview of recent progress of the NAA methods, mainly highlighting the PCR assay technique, was presented. In particular, the innovation of nested PCR assay technique is worthy of note given its contribution to improve the diagnosis of CNS tuberculosis. Although a novel assay technique, which is internally controlled and combines the high sensitivity of nested PCR with the accurate quantification of real-time (TaqMan) PCR, namely, “WR-QNRT-PCR assay,” is reported as a rapid diagnostic method in TBM, it is not widely used in routine clinical examination. This novel assay technique with high sensitivity in addition to accurate quantification is useful for not only the rapid diagnosis of CNS tuberculosis but also the prediction of prognosis and assessing the effect of ATT during the clinical course of TBM. Therefore, in actual clinical practice, its wider use for the diagnosis of CNS tuberculosis is expected in the future.

Disclosure

All authors have not received any financial support from anywhere, with regard to this paper. All authors have agreed and approved submission of this paper to this journal.

Conflict of Interests

There is no conflict of interest in connection with this paper.

References


Clinical Study
Maintenance of Sensitivity of the T-SPOT.TB Assay after Overnight Storage of Blood Samples, Dar es Salaam, Tanzania

Elizabeth A. Talbot,1,2 Isaac Maro,3 Katherine Ferguson,4 Lisa V. Adams,1 Lillian Mtei,3 Mecky Matee,3 and C. Fordham von Reyn1

1 Dartmouth Medical School, Hanover, NH 03755, USA
2 Infectious Diseases and International Health Section, 1 Medical Center Drive, Lebanon, NH 03756, USA
3 Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania
4 Dartmouth College, Hanover, NH 03755, USA

Correspondence should be addressed to Elizabeth A. Talbot, elizabeth.talbot@dartmouth.edu

Received 25 November 2011; Accepted 15 January 2012

Academic Editor: Catharina Boehme

Copyright © 2012 Elizabeth A. Talbot et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background. T-SPOT.TB is an interferon gamma release assay for detecting Mycobacterium tuberculosis infection. The requirement to process within 8 hours is constraining, deters use, and leads to invalid results. Addition of T Cell Xtend reagent may allow delayed processing, but has not been extensively field tested. Design. Consecutive AFB smear positive adult tuberculosis patients were prospectively recruited in Dar es Salaam, Tanzania. Patients provided a medical history, 1–3 sputum samples for culture and 1 blood sample which was transported to the laboratory under temperature-controlled conditions. After overnight storage, 25 μL of T Cell Xtend reagent was added per mL of blood, and the sample was tested using T-SPOT.TB. Results. 143 patients were enrolled: 57 patients were excluded because temperature control was not maintained, 19 patients were excluded due to red blood cell contamination, and one did not provide a sputum sample for culture. Among 66 evaluable patients, overall agreement between T-SPOT.TB and culture was 95.4% (95%CI; 87.1–99.0%) with Kappa value 0.548. Sensitivity of T-SPOT.TB when using T Cell Xtend reagent was 96.8% (95%CI; 88.8–99.6%). Conclusions. When T Cell Xtend reagent is added to specimens held overnight at recommended temperatures, T-SPOT.TB is as sensitive as the standard assay in patients with tuberculosis.

1. Introduction
The T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK) is an interferon gamma release assay (IGRA) for detection of latent Mycobacterium tuberculosis infection (LTBI). This assay detects T-lymphocytes specific for M. tuberculosis antigens that are absent from M. bovis BCG and most environmental mycobacteria [1]. Currently, T-SPOT.TB must be performed within 8 hours of sample collection. Logistically, this requires that patients have blood drawn early enough to transport the sample to the laboratory, which must assay samples on the day of receipt. This precludes batching samples, which would save resources and testing supplies. Moreover, in many countries, laboratories are centralized and are not in close proximity to where the blood sample is drawn. Therefore, it is not possible to transport samples to arrive at the receiving laboratory for processing on the same day.

To allow greater test processing flexibility, the manufacturer of T-SPOT.TB developed a proprietary reagent called T Cell Xtend, which they report can be added to whole blood samples stored at 10–25°C to increase this timeframe up to 32 hours [2].

2. Materials and Methods
The ethical review boards of Dartmouth Medical School (Hanover NH), the National Institute for Medical Research...
2.3. Mycobacterial Studies. AFB smears were done according to the Ziehl-Neelsen method by trained research personnel. Sputum was cultured for mycobacteria on Lowenstein Jensen slants.

2.4. Statistical Analyses. Sensitivity was calculated as the number of T-SPOT.TB positive samples divided by the number of culture positive samples multiplied by 100. 95% confidence limits were calculated. The Kappa statistic was used to measure overall agreement between culture and T-SPOT.TB results. Analyses were conducted using Excel.

3. Results

A total of 143 participants were enrolled into the study (Figure 1). The initial 57 blood samples drawn at one clinic were transported and stored at ambient temperatures prior to processing. After processing, it was recognized that these specimens were not reliably kept at temperatures below 25°C (the required temperature per protocol), and therefore these 57 samples were excluded from the final sensitivity analysis. Temperature control measures were instituted for the subsequent collection of samples from an additional 86 participants. Nineteen of these 86 (22.1%) participants were excluded because the laboratory observed red blood cell contamination in the PBMC layer so the PBMCs could not be accurately counted. One of 86 (1.2%) participants failed to provide a sputum sample for culture and was excluded.

Therefore, 66 results were available for analysis. The participants were all black African and ranged in age from 18–60 years. The majority were male and BCG vaccinated (71% and 77%, resp.); 17 (26%) were HIV-positive, 24 (36%) were HIV-negative, and 25 (38%) declined testing.

Of the 66 T-SPOT.TB results, 61 (92.4%) were positive, 4 (6.1%) were negative, and 1 (1.5%) was invalid. The overall agreement between the 65 valid T-SPOT.TB and culture results was 95.4% (62/65: 95%CI; 88.8–99.6%) with a Kappa value of 0.548. The sensitivity of the T-SPOT.TB assay when run after delayed processing using the T Cell Xtend reagent was 96.8% (60/62: 95%CI; 88.8–99.6%). Among 41 T-SPOT.TB results from participants with known HIV status, T-SPOT.TB results were positive in 14 (82.4%) of those who were HIV-positive and 23 (95.8%) of those who were HIV-negative (P > 0.05). However, limiting to valid results from culture positive patients, the sensitivity of the assay among samples from HIV-positive participants was 92.9% (13/14).

In spite of exclusion because of protocol noncompliance, results from the 57 samples transported at ambient temperature were also analyzed. Of the 57, 40 (70.2%) were positive, 11 (19.3%) were negative, and 6 (10.5%) was invalid. Compared with the results from the 66 samples with temperature control, false negative results among these 57 samples were more common (P = 0.05). Results from 19 samples were excluded because of red blood cell contamination showed 14 (73.7%) positive and 5 (26.3%) negative. Compared with the results from 66 samples with temperature control, false negative results among these 19 were statistically significantly more common (P = 0.04).

4. Discussion

T-SPOT.TB with delayed processing using T Cell Xtend among patients with culture-confirmed tuberculosis showed high sensitivity, comparable to that reported for immediate
Figure 1: Patient flow and testing results using T-SPOT.TB and T Cell Xtend, Dar es Salaam, Tanzania.

Table 1: Overall agreement between TB culture and T-SPOT.TB.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Positive</th>
<th>Negative</th>
<th>Invalid</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SPOT.TB</td>
<td>60</td>
<td>2</td>
<td>1*</td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*1/66 (1.5%) of T-SPOT.TB results was invalid due to a high nil control result.

5 Conclusions

T-SPOT.TB can be run on blood samples not contaminated with red blood cells and maintained overnight by the use of T Cell Xtend reagent without affecting the sensitivity of the assay. Storage and transport of samples at temperatures...
<25°C are critical to obtaining optimum sensitivity, and use of an automated cell counting instrument may correct the reduced sensitivity observed with red blood cell contamination.

Authors’ Contributions

E. A. Talbot, L. V. Adams, L. Mtei, and C. F. von Reyn designed the study. E. A. Talbot, I. Maro, K. Ferguson, L. V. Adams, L. Mtei, M. Matee, and C. F. von Reyn participated in the implementation of the trial. E. A. Talbot, L. Mtei, M. Matee, and C. F. von Reyn participated in administration. I. Maro, K. Ferguson, and L. Mtei participated in data collection. E. A. Talbot analyzed the data and wrote the first draft. E. A. Talbot, L. V. Adams, L. Mtei and C. F. von Reyn contributed to the interpretation of the data. All authors reviewed and approved the final paper.

Acknowledgments

The authors would like to express their gratitude to the volunteers who participated in this study. They appreciate the efforts of the clinical research staff: Dr. Ibrahim Mteza, Esther Kayichile, Joyce Wamsele, and Emmanuel Balandya. They also thank Betty Mchaki (DarDar Project) and Wendy Wieland-Alter (Dartmouth Medical School) for their generous laboratory assistance and Miriam Zayumba (DarDar Project) and Susan Tvaroha (Dartmouth College) for assistance with data management. The authors disclose that Oxford Immunotec, the manufacturer of the T-SPOT.TB and the T Cell Xtend reagent, funded this study and also contributed in on-site study monitoring. Oxford Immunotec did not interpret the findings or write this paper. C. F. von Reyn has been a one-day consultant for Oxford Immunotec on two occasions, and C. F. von Reyn, L. V. Adams, and E. A. Talbot received additional research funding from Oxford Immunotec more than one year prior to this submission. The other authors have no potential conflicts to declare.

References


Clinical Study
The Use of Interferon Gamma Release Assays in the Diagnosis of Active Tuberculosis

Silvan M. Vesenbeckh,1 Nicolas Schönfeld,1 Harald Mauch,2 Thorsten Bergmann,2 Sonja Wagner,2 Torsten T. Bauer,1 and Holger Rüssmann2

1Department of Pneumology, Lungenklinik Heckeshorn, HELIOS Klinikum Emil von Behring, 14165 Berlin, Germany
2Institute of Microbiology, Immunology and Laboratory Medicine, HELIOS Klinikum Emil von Behring, 14165 Berlin, Germany

Correspondence should be addressed to Silvan M. Vesenbeckh, vesenbeckh@gmail.com

Received 25 November 2011; Revised 5 January 2012; Accepted 6 January 2012

Interferon gamma release assays (IGRAs) are in vitro immunologic diagnostic tests used to identify *Mycobacterium tuberculosis* infection. They cannot differentiate between latent and active infections. The cutoff suggested by the manufacturer is 0.35 IU/mL for latent tuberculosis. As IGRA tests were recently approved for the differential diagnosis of active tuberculosis, we assessed the diagnostic accuracy of the latest generation IGRA for detection of active tuberculosis in a low-incidence area in Germany. Our consecutive case series includes 61 HIV negative, *Mycobacterium tuberculosis* culture positive patients, as well as 234 control patients. The retrospective analysis was performed over a period of two years. In 11/61 patients with active tuberculosis (18.0%) the test result was <0.35 IU/mL, resulting in a sensitivity of 0.82. We recommend establishing a new cutoff value for the differential diagnosis of active tuberculosis assessed by prospective clinical studies and in various regions with high and low prevalence of tuberculosis.

1. Introduction

Tuberculosis (TB) remains a major public health problem affecting one-third of the world’s population [1, 2]. Diagnosis of TB is usually based on a combination of anamnestic symptoms, clinical presentation, radiological and pathological changes, bacteriological findings of acid/alcohol-fast bacilli, and molecular tests [3]. Definitive TB diagnosis is based on the detection of *Mycobacterium tuberculosis* (MTB) in the culture, which usually takes four to six weeks. For decades, tuberculin skin test (TST) has been used as diagnostic tool to support the physician’s decision process. With the introduction of interferon gamma release assays (IGRAs), a more specific method became available. Although primarily developed for the diagnosis of latent TB, clinicians have also been searching for improved diagnostic tools and explored IGRAs for the immunodiagnosis of active TB. In 2010, the Centers for Disease Control and Prevention (CDC) updated their guidelines for testing for TB infection, concluding that IGRAs “may be used instead of a tuberculin skin test in all situations in which the CDC recommends the tuberculin skin test as an aid in diagnosing *M. tuberculosis* infection” [4, 5].

Nevertheless, with the cutoff for the diagnosis of latent TB as given by the producers, pooled sensitivity for the diagnosis of culture positive TB did not exceed 80% in the most recent meta-analyses [6, 7]. The present case series constitutes one of the largest reports of latest generation IGRA used in culturally proven HIV-negative TB cases in a low-prevalence country. Our study is meant to help evaluate the cutoff for the IGRA in the differential diagnosis of TB.

2. Study Population and Methods

This is a retrospective study performed on inpatients of a regional hospital specialized in lung diseases (Lungenklinik Heckeshorn, Berlin). At least one IGRA is routinely performed on a blood sample of each TB-suspect patient, and every suspicious sample (smear, lymph node biopsy, pleural effusion, or biopsy) is routinely cultured for TB. All
Table 1: (a) Median and range for patient age and IGRA test result for all culture positive cases of active tuberculosis, by organ manifestation. (b) Median and range for age and IGRA test result for all controls, by diagnosis. “Others” includes other lung diseases such as asbestosis, aspergillosis, asthma, bronchiectasis, pleural effusion, haemoptysis, fibrosis, and sarcoidosis. (c) Study results for TB cases and controls. (n: sample size, CI: confidence interval).

<table>
<thead>
<tr>
<th>(a) MTB infection</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>Median age, years (range)</th>
<th>Median QFT, IU/mL (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>28</td>
<td>25</td>
<td>53</td>
<td>45 (4–83)</td>
<td>2.40 (0.00–103.4)</td>
</tr>
<tr>
<td>Pleura</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>26 (17–76)</td>
<td>2.45 (0.96–300)</td>
</tr>
<tr>
<td>Lymph node</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>65 (23–69)</td>
<td>21.32 (3.78–86.8)</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>29</td>
<td>61</td>
<td>46 (3–84)</td>
<td>3.46 (0.00–300)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Diagnosis</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>Median age, years (range)</th>
<th>Median QFT, IU/mL (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignancy</td>
<td>22</td>
<td>23</td>
<td>45</td>
<td>69 (39–90)</td>
<td>0.01 (0.00–23.9)</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>27</td>
<td>13</td>
<td>40</td>
<td>64 (1–90)</td>
<td>0.00 (0.00–1.84)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>30</td>
<td>17</td>
<td>47</td>
<td>67 (1–99)</td>
<td>0.00 (0.00–75)</td>
</tr>
<tr>
<td>Others</td>
<td>53</td>
<td>49</td>
<td>102</td>
<td>62.5 (5–99)</td>
<td>0.02 (0.00–103.4)</td>
</tr>
<tr>
<td>Total</td>
<td>132</td>
<td>102</td>
<td>234</td>
<td>66 (1–99)</td>
<td>0.00 (0.00–103.4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(c) n QFT Age</th>
<th>Mean</th>
<th>95% CI</th>
<th>P</th>
<th>Mean</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>61</td>
<td>14.13</td>
<td>3.44–24.82</td>
<td>&lt;0.05</td>
<td>47.8</td>
<td>41.88–53.79</td>
</tr>
<tr>
<td>Control</td>
<td>234</td>
<td>1.33</td>
<td>0.24–2.43</td>
<td></td>
<td>63</td>
<td>60.56–65.39</td>
</tr>
</tbody>
</table>

samples of patients included in this study were taken prior to initiation of antibiotic therapy. Over a two-year period (1/2008–1/2010), IGRA results of all MTB culture positive cases of active TB were analyzed. Patients with other lung diseases than TB, including negative history for MTB infection and without radiological findings suggestive of MTB infection in the past and with no signs of active disease, were chosen as control group. All HIV-positive patients were excluded from the database.

2.1. IGRA. We used the latest generation IGRA (Quantiferon-TB Gold in-Tube, Cellestis, Carnegie, Australia), later referred to as QFT-GIT, on all samples. Peripheral blood samples were obtained by trained personnel in specific blood collection tubes following the manufacturer’s instructions. All blood samples were processed within 4 h of phlebotomy. Otherwise, the test was performed as previously described [8].

2.2. TB Culture. Specimens were stained, processed, and cultured by standard procedures in mycobacteriology [9]. The isolates were cultured for 4 weeks on Löwenstein-Jensen (L-J) medium at 37°C and tested for growth rate, pigment production, and by biochemical testing using standard methods [10, 11].

2.3. Statistical Analysis. Data were analyzed using STATA 12.0 (StataCorp, College Station, Texas, USA). The t-test for independent samples (two-tailed) was carried out to assess significance level of detected differences in both groups (IGRA test results, age).

3. Results

A total of 61 patients with active TB as ascertained through positive MTB culture were examined using QFT-GIT between 1/2008 and 1/2010 (see Table 1(a)). 53 patients presented with pulmonary TB, three patients with lymph node TB, and five patients with tuberculous pleurisy. The median QFT-GIT value for all TB patients was 3.46 IU/mL, ranging from 0.00 to 300 IU/mL. In 11 patients, the test result was <0.35 IU/mL (see Figure 1). Assuming the cutoff for latent TB suggested by the producer, these are false-negative (FN) test results (11/61 = 18% FN). We calculated the sensitivity (the proportion of patients with active TB who are correctly identified as such) as 1-FN (82%). Only in one patient four months after therapy with adalimumab, the result was 0.00 IU/mL, and in one other patient with lung cancer under concomitant chemotherapy, it was 0.02 IU/mL. All other patients showed values of 0.07 IU/mL or above.

The median age of our patients was 46 years (range: 5–84 years), in the group with a test result ≥0.07 and <0.35 IU/mL, the median age was 80 years (range: 44–84 years), and, in the group testing ≥0.35 IU/mL, it was 36 years (range: 5–84 years).

Within the control group (see Table 1(b)), QFT-GIT values ranged from 0.00 to 103.4 IU/mL in one patient with pneumonia. The median was 0.00 IU/mL. In 50% of all cases (116/234), no Interferon gamma (IFN-γ) release was measured (0.00 IU/mL). In 51/234 patients (21%), test results
were above 0.35 IU/mL. The median age in the control group was 66 years with a range from 1 to 99 years.

Patients in the study group were significantly younger than in the control group and had significantly higher QFT-GIT test results (P < 0.05 for both, see Table 1(c)).

4. Discussion

IGRAs are in vitro immunologic diagnostic tests to identify MTB infection. Latent and active infections are not differentiated. Several test systems are commercially available: QuantiFERON-TB Gold (QFT), QuantiFERON-TB Gold in-Tube (QFT-GIT), both Cellestis Limited, Chadstone, Australia, and T-SPOT.TB (Oxford Immunotec, Abingdon, UK). The QFT-GIT measures IFN-γ responses to the MTB specific antigens early secretory antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), and Rv2654 (TB 7.7). Their use in clinical practice is more and more widespread. Whereas the test was initially conceived to support diagnosis of latent infection, an increasing body of evidence is published on its use in detection of active TB infection [12–14]. More and more guidelines now include recommendations for or against the use of IGRAs in the differential diagnosis of active TB [15].

Several systematic reviews and meta-analyses have recently been published specifically on the diagnostic accuracy of IGRAs in active TB [6, 16, 17]. Strong heterogeneity between study populations is a major limitation of these meta-analyses, and most studies had small sample sizes. Overall, few studies were done with the latest generation QFT-GIT in areas of low endemicity such as Germany (2 studies in [17], 13 studies in [16]). Among those, even fewer restricted sensitivity analysis to patients with culturally proven MTB infection and confirmed HIV-negative status [12, 18].

Specificity depends highly on the definition of the control group. Pai et al. reported a pooled specificity of 99% among non-BCG vaccinated and 96% among BCG-vaccinated low-risk groups [17]. According to a recent meta-analysis that did not restrict studies on specificity to low-risk groups [6], a situation more compatible to our clinical setting, the specificity of QFT-GIT was only 0.79 (95% CI 0.75–0.82). In our study, 51 out of 234 control patients (21%) showed test results above 0.35 IU/mL, indicative of latent TB according to the producer. However, this finding is also consistent with the expected number of false positives assuming a specificity of around 0.8 according to Sester et al. [6]. The retrospective design of our study does not allow any conclusion about the test specificity in our study population.

Sensitivity in these studies was also found to be highly dependent on the study population, notably local TB prevalence, and ranged from 0.58 in a high-prevalence country [19] to 1.00 in a low-prevalence country [20], when QFT-GIT was assessed. Diel et al. found a pooled sensitivity of 0.84 (95% CI 0.81–0.87) when including only developed countries [16], consistent with the results published by Sester et al. (0.77, 95% CI 0.75–0.80) [6].

In our consecutive case series of 61 TB culture positive, HIV-negative patients in a low-prevalence setting in Germany, we found a sensitivity of 82.0% (95% CI: 0.696, 0.902) for the QFT-GIT, when the cut-off recommended for the diagnosis of latent TB was used (<0.35 IU/mL). In a low-prevalence country such as Germany with a TB prevalence of 5.4/100,000 inhabitants [21], case finding is an outstanding priority in the management of this disease. Therefore, highly sensitive test systems are needed for screening purposes. If the use of IGRA in the differential diagnosis of active TB is recommended, at least under certain conditions, then the cut-off point for active TB should be lower compared to latent TB, as previously discussed by Davidow [22]. Prospective clinical studies in different defined regions, with high- and low-prevalence of active TB, should be performed to evaluate the use of IGRAs in the diagnostic workup of TB patients. The cutoff for the detection of latent TB does not seem applicable for that purpose.

5. Conclusions

We suggest that the cutoff for the use of IGRA in the differential diagnosis of active TB in low incidence settings be reevaluated. Further prospective studies including clinical criteria for TB are needed to determine a new cutoff for active TB.

Authors’ Contribution

H. Mauch, N. Schönfeld, and S. M. Vesenbeckh designed the study, T. Bergmann and S. Wagner managed data acquisition and compilation, N. Schönfeld and S. M. Vesenbeckh analysed the data, T. T. Bauer and S. M. Vesenbeckh did statistical
analysis, S. M. Vesenbeckh wrote the paper, and T. T. Bauer, H. Mauch, H. Rüssmann, and N. Schönfeld reviewed and edited the manuscript.

Funding
This work was funded by HELIOS Klinikum Emil von Behring and received support by DZK (Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose) and OHH (Stiftung Oskar-Helene-Heim).

Conflict of Interests
The authors declare that there is no conflict of interests.

References
Clinical Study

Comparison of Overnight Pooled and Standard Sputum Collection Method for Patients with Suspected Pulmonary Tuberculosis in Northern Tanzania

Stellah G. Mpagama,1, 2 Charles Mtabho,2 Solomon Mwaigwisya,2 Liberate J. Mleoh,1 I Marion Sumari-de Boer,2 Scott K. Heysell,3 Eric R. Houpt,3 and Gibson S. Kibiki2

1 Kibong’oto National Tuberculosis Referral Hospital, P.O. Box 12, Kilimanjaro, Tanzania
2 Kilimanjaro Clinical Research Institute and Kilimanjaro Christian Medical College, Kilimanjaro Mashi, Tanzania
3 Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA 22908, USA

Correspondence should be addressed to Stellah G. Mpagama, s.mpagama@kcri.ac.tz

Received 28 September 2011; Accepted 3 January 2012

Academic Editor: Catharina Boehme

Copyright © 2012 Stellah G. Mpagama et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In Tanzania sputum culture for tuberculosis (TB) is resource intensive and available only at zonal facilities. In this study overnight pooled sputum collection technique was compared with standard spot morning collection among pulmonary TB suspects at Kibong’oto National TB Hospital in Tanzania. A spot sputum specimen performed at enrollment, an overnight pooled sputum, and single morning specimen were collected from 50 subjects and analyzed for quality, quantity, and time to detection in Bactec MGIT system. Forty-six (92%) subjects’ overnight pooled specimens had a volume ≥ 5 mls compared to 37 (37%) for the combination of spot and single morning specimens (P < 0.001). Median time to detection was 96 hours (IQR 87–131) for the overnight pooled specimens compared to 110.5 hours (IQR is 137 right 137–180) for the combination of both spot and single morning specimens (P = 0.001). In our setting of limited TB culture capacity, we recommend a single pooled sputum to maximize yield and speed time to diagnosis.

1. Background

Tuberculosis (TB) and HIV are among the global leaders in infectious disease mortality [1]. Sub-Saharan Africa has one of the highest burdens of TB and HIV coinfection [2]. Prompt diagnosis of TB is critical to improve outcome, but diagnosis of TB is challenging in HIV-infected patients and especially in resource-limited settings [3]. HIV-infected patients have a higher rate of extrapulmonary TB, atypical chest radiographs and fewer pulmonary cavities [4–6]. As a consequence, HIV patients may be more likely to have a negative or paucibacillary sputum smear microscopy [7]. Subjects with paucibacillary specimens may be prone to being delayed in clinical diagnosis, either because acid-fast bacilli are not observed by microscopy or time to detection Mycobacterium tuberculosis (MTB) culture is prolonged [8]. Both poor quality and low quantity of sputum have a significant impact on TB detection rate [9, 10]. In settings reliant on smear microscopy as the only means of TB diagnosis, this may impact negatively the time to initiation of TB treatment. Ideal specimens should contain 5 mL or more of sputum without saliva. A previous study found that the quality and quantity of sputum were improved by pooling three versus a single “spot” collection [11]. However, collection on multiple days may unnecessarily burden health facilities and may be prone to contamination. In contrast, a single overnight pooled technique whereby a patient is given a sealable container in which to collect all expectorated sputum over the course of the night has been used in TB treatment trials and in assessment of early bactericidal activity of new TB medications [12, 13]. However it has not been examined in routine clinical practice.

At Kibong’oto National TB Hospital in Kilimanjaro, Tanzania, HIV co infection among TB suspects is common and it
Enrolled: 50 subjects
All subjects produced one of each specimen:
(1) a spot specimen \( (n = 50) \)
(2) an overnight pooled specimen \( (n = 50) \)
(3) a follow-up morning specimen \( (n = 50) \)

All specimens analyzed for quality: comparison was made between overnight pooled specimen and the combination of spot and follow-up morning specimens

All specimens split for Ziehl-Neelsen microscopy, solid culture, and liquid culture (Bactec MGIT): comparison of microscopy, culture yield, colony-forming unit count on solid culture, and time to detection in liquid culture was made between the overnight pooled specimen and both the spot specimens and follow-up morning specimens

Figure 1: Study diagram of enrollment and specimen analysis.

Table 1: Demographic and clinical characteristics.

<table>
<thead>
<tr>
<th>Variables (( N = 50 ))</th>
<th>Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36 (72%)</td>
</tr>
<tr>
<td>Age (years) median (IQR)</td>
<td>38.5 (30–46)</td>
</tr>
<tr>
<td>Weight (kg) median (IQR)</td>
<td>51 (47–58)</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>CD4 count (cells/( \mu L )) by HIV status in median (IQR)</td>
<td></td>
</tr>
<tr>
<td>HIV positive</td>
<td>71 (30.5–158)</td>
</tr>
<tr>
<td>HIV negative</td>
<td>540 (409–614)</td>
</tr>
<tr>
<td>Chest X-ray findings</td>
<td></td>
</tr>
<tr>
<td>Cavities</td>
<td>23 (46%)</td>
</tr>
</tbody>
</table>

has been observed that the minority of patients have cavitary chest radiographs. Furthermore it is a standard practice that each TB suspect is given a sealable mug to collect all sputum as an infection control measure. Thus, among a population at risk for lower bacillary yield, we sought to compare overnight pooled sputum in the sealable mugs to the standard spot technique for sputum quality, quantity, and time to MTB detection by culture in the Bactec MGIT system, a method which has correlated well with conventional colony counts [14, 15].

2. Methods

Subjects suspected of pulmonary TB at Kibong’oto National TB Hospital were recruited for enrollment. Subjects were eligible if they were 18 years and above and presenting with two weeks or more of cough. Each patient was asked to collect three sputum samples in calibrated wide mouthed container: (1) a spot specimen at the time of enrollment, (2) an overnight pooled specimen, and (3) a spot specimen on the morning after enrollment (Figure 1). Subjects were excluded if initiated on any antituberculosis medication prior to sample collection. Sputum samples were sent to Kili- manjaro Clinical Research Institute (KCRI) laboratory for processing. The specimens were assessed for volume collected, presence of blood, and color. Samples were analyzed for quality based on the following criteria: (1) volume equal to or more than 5 mls, (2) sputum color was white, yellow, green, or red, (3) presence of blood, and (4) thick consistency. Each sputum specimen was decontaminated with 4% NaOH and centrifuged at 1500 g for 10 minutes. The sediment was split equally for use in direct smear microscopy by Ziehl-Neelsen staining, MTB culture on solid agar with Lowenstein-Jensen slant and Middlebrook 7H11 media, and in liquid media using Bactec MGIT 960 machine (Becton Dickinson, USA). Positive culture growth was confirmed by secondary Ziehl-Neelsen stain. Colony-forming unit (CFU) count was performed on the Middlebrook 7H11 media.

Data were analyzed by Stata Version 11 statistical software (StataCorp, U.S.A). Values were presented as means with standard deviation (SD) or median with interquartile range (IQR) for data that was not normally distributed. Chi-square was used for dichotomous variables and Spearman’s correlation coefficient for continuous variables to investigate the association with microscopy and culture yield. All tests were two sided with \( P \) values \( \leq 0.05 \) regarded as statistically significant.

All subjects provided written informed consent. Ethical approval for the study was given by the IRB of the Kilimanjaro Christian Medical Center, Tumaini University and the National Ethical Review Board.

3. Results

Fifty pulmonary TB suspects were enrolled for the study, each produced three specimens, hence 150 samples were used for analysis. The median age of patients was 38.5 years (IQR 30–45) (Table 1). Thirty-six (72%) were male and 8 (16%)
Table 2: Comparison of overnight pooled and spot method of collection on quality and quantity of sputum.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Combination of spot and single morning specimen</th>
<th>Overnight pooled</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity (≥5 mls)</td>
<td>37/100 (37%)</td>
<td>46/50 (92%)</td>
<td><em>P</em> &lt; 0.001</td>
</tr>
<tr>
<td>Color</td>
<td>92/100 (92%)</td>
<td>47/50 (94%)</td>
<td><em>P</em> = 0.79</td>
</tr>
<tr>
<td>Thick consistency</td>
<td>66/100 (66%)</td>
<td>41/50 (82%)</td>
<td><em>P</em> = 0.12</td>
</tr>
<tr>
<td>Presence of blood</td>
<td>11/100 (11%)</td>
<td>6/50 (12%)</td>
<td><em>P</em> &gt; 0.99</td>
</tr>
</tbody>
</table>

*P* values are similar if pooled is compared with spot or single morning specimen, thus we combined spot and single for this table.

were HIV-1-infected patients. Among the HIV-infected subjects, the median CD4 count was 71 cells/µL (IQR 30.5–158). The median CD4 count for HIV-seronegative patients was 540 cell/µL (IQR 409–614). Twenty-three (46%) had cavitary disease (Table 1).

Overnight pooled sputum yielded a specimen of ≥5 mL in 46 (92%) of subjects compared to 37 (37%) for the combination of spot and single morning specimens (*P* < 0.001). Good quality of sputum specimen by color was observed in 47 (94%) of the patients for overnight pooled sputum—while combination of spot and single morning specimens was 92 (92%) (*P* = 0.79). Thick consistency was observed in 41 (82%) of patients for overnight pooled compared to combination of spot and single morning 66 (66%) (*P* = 0.12), and presence of blood was observed in 6 (12%) and 11 (11%), respectively (*P* > 0.99) (Table 2).

Overall 100 (67%) specimens were positive by smear microscopy. Thirty-four (68%) spot specimens were smear positive, compared to 31 (62%) single morning specimens, and 35 (70%) for overnight pooled (*P* = 0.28). Eighty-one (54%) culture-positive specimens were available for comparison of time to detection in the MGIT system from 27 subjects; 49 (33%) specimens were negative and bacterial contamination precluded analysis in 20 (13%). The MGIT contamination rate did not differ from that of Lowenstein-Jensen, found in 21 (14%) of all specimens. Furthermore, among MGIT specimens contamination did not vary by method of collection: spot (5.3%), single morning (3.3%) and overnight pooled (4.7%) (*P* > 0.99). However, the median time to detection was 96 hours (IQR 87–131) for the overnight pooled specimens which was significantly faster compared to 118 hours (IQR 98–167) for single morning specimen and 143 hours (IQR 108–194) for the spot specimens (*P* < 0.005) (Figure 2).

Only a limited number of pooled specimens were TB-culture positive on Middlebrook 7H11 to allow colony forming unit (CFU) count determination. The median CFU count among subjects with cavities on chest radiograph was 4.92 log/mL (IQR 4.3–6.28) compared to 3.95 log/mL (IQR 3.61–4.84) in patients without cavities (*P* = 0.12). Among the 8 HIV-infected subjects, only 2 (25%) had overnight pooled specimens available for CFU count. The median CFU count among HIV-infected subjects was 4.34 log/mL (IQR 3.9–4.72) compared to 4.84 log/mL (IQR 4.30–5.86) for the HIV-uninfected subjects (*P* = 0.43). There was no correlation between CD4 counts with CFU (Spearman’s rho = −0.36) (*P* = 0.16).

4. Discussion

The major finding of this study was that in routine practice an overnight pooled sputum collection significantly improved the time to detection in the MGIT system among culture-positive samples. This is an important finding amidst the global scale-up of MTB culture.

Faster time to diagnosis in a TB suspect can speed drug susceptibility testing, hasten proper treatment, and potentially decrease transmission to the community and within the hospital. In settings with increasing rates of multidrug-resistant (MDR) TB, faster drug susceptibility results have been postulated to be one of the most important means of improving MDR TB outcome [16].

Sputum collection is the first and most critical step in laboratory diagnosis of pulmonary TB. In resource-limited settings such as ours, multiple specimen collection has been proposed to increase yield for rapid molecular probe diagnostics [17, 18]; however it can be overwhelming to the laboratory. Both microscopy and culture are time consuming and culture is costly. In Tanzania culture is recommended for smear-negative cases, for treatment failures and for monitoring MDR-TB patients. Currently there are few laboratories capable of culture and specimens must be sent long distances. In this setting, maximizing yield by sending the optimal specimen will conserve resources. We would strongly advocate sending pooled specimens in our context.

Our findings of increased culture yield with volumes of a minimum of 5 mls of adequate quality are consistent with previous studies that examined yield of microscopy [19, 20].
However, our microscopy yield did not approach the remarkable >95% rate of smear positivity reported by others when a 24-hour collection technique was used for diagnosis of selected subjects with pulmonary TB [21]. This is potentially contributed by the fact that we selected for pulmonary TB suspects and not confirmed cases. With an eye towards molecular diagnostics in the future, given the drop in sensitivity of the rapid molecular diagnostics in smear-negative sputum samples [17], we would expect that overnight pooled sputum collection would be equal to or improve upon molecular diagnostic yield compared with a spot specimen.

Our study has several limitations. For the CFU analysis on the overnight pooled specimens, fungal overgrowth limited examination of nine different plates. The contamination affected subjects with and without cavities so it is unlikely to have biased this finding; yet overall our total number of patients enrolled was small and may not have included enough HIV-infected patients to appreciate differences in this subgroup with regard to culture yield, CFU analysis, and time to detection. Bacterial contamination also limited evaluation of all samples in the MGIT, but the contamination was observed in all specimen collection techniques and has been reported in other evaluations of liquid culture in MGIT [22]. Furthermore, the culture yield for all techniques may have been diminished by splitting specimens for both solid and liquid culture.

In conclusion, we found that overnight pooled sputum collection improved the quantity of sputum without alteration in quality and led to faster time to detection. These findings can ultimately impact the decisions both of treatment and infection control. Overnight pooled sputum should be considered as an alternative to standard spot testing in resource-limited settings.

Acknowledgments

The authors would like to acknowledge the African Poverty Related-Infection Oriented Research Initiative (APRIORI) which supported grant of training in master degree of clinical research of which S. G. Mpagama is a recipient. S. G. Mpagama is also supported by the NIH Fogarty’s Global Infectious Disease Research Training Program (D43 TW008270) which enabled final drafting of the paper.

References


