Comparison of the automated Cobas Amplicor *Mycobacterium tuberculosis* assay with the conventional methods for direct detection of *Mycobacterium tuberculosis* complex in respiratory and extrapulmonary specimens

Mubarak S. Alfaresi, *MBBS, FACHARZT,* ***Adeel I. Abdulsalam,*** *MSc, M.Phil,* ***Abida A. Elkoush,*** *Bch.*

ABSTRACT

Objective: To evaluate the recently available Cobas Amplicor polymerase chain reaction (PCR) system for the detection of *Mycobacterium tuberculosis* complex (MTBC) in well-characterized clinical specimens and to compare the results with clinical classification, conventional culture and staining techniques.

Methods: Three hundred and forty-four clinical specimens consecutively received for culture of acid-fast bacilli by the laboratory of Zayed Military Hospital, Abu Dhabi, United Arab Emirates, from 2004 to 2005, were used in this study. Acid-fast bacilli and culture for tuberculosis were compared with Cobas Amplicor PCR.

Results: The final diagnostic evaluation of the Cobas Amplicor MTB showed the followings: sensitivity 73.3%, specificity 99.4%, positive predictive value 84.6%, negative predictive value 98.8% and with accuracy of 98.2% for the Cobas Amplicor MTB assay.

Conclusion: The automated Cobas Amplicor MTB assay is suitable for routine use in clinical laboratories.

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One-third of the world's population has been infected by *Mycobacterium tuberculosis* (*M. tuberculosis*), and there are approximately 8 million new cases of tuberculosis (TB) annually. The incidence of TB is constantly increasing, and strains of *M. tuberculosis* resistant to chemotherapeutic agents have been recovered with increasing frequency.[1] The World Health Organization declared TB as a global health emergency in 1993, as the genetic variability of *M. tuberculosis* is relevant for the epidemiology of TB, which is still the most significant bacterial disease of human's worldwide.[2]

Conventional methods include the acid-fast stain, culture, and biochemical tests for detecting and identifying members of the *M. tuberculosis* complex (MTBC). Even with concentrated samples, the sensitivity of microscopy is not great, and *M. tuberculosis* cannot be reliably differentiated from other mycobacteria in acid-fast-stained smears. Although the use of fluorescent (auramine-rhodamine) stains increases the sensitivity and shortens the time required for reading of smears, microscopy, as a direct method, can provide at best only a preliminary diagnosis.[3,4]

From the Department of Microbiology, Zayed Military Hospital, Abu Dhabi, *United Arab Emirates*.

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Address correspondence and reprint request to: Dr. Mubarak S. Alfaresi, Clinical Microbiologist and Infectious Diseases Epidemiologist, Department of Pathology and Laboratory Medicine, Microbiology Division, Zayed Military Hospital, Abu Dhabi, *United Arab Emirates*. E-mail: uaenow@emirates.net.ae

Cultural methods, properly applied, detect *M. tuberculosis* in clinical samples with reasonable sensitivity and provide accurate identification of the isolates. However, these methods are quite slow, requiring 3-8 weeks for completion, primarily due to the slow growth of the mycobacteria.[5,6] Once the presence of mycobacteria is indicated, additional testing is required to identify the species. There is thus an urgent need for a rapid, safe, and verifiable method to establish the diagnosis of TB. Substantial changes in methodology have occurred with the widespread use of non-radioactive DNA probes for culture identification and most recently the application of highly sensitive in vitro nucleic acid amplification techniques for the direct detection and specific identification of mycobacteria in clinical specimens.[7-10] Although a number of amplification methods, such as ligase chain reaction, strand displacement amplification and transcription-based amplification, have been developed in the emerging field of molecular diagnostics the original and most widely applied amplification method is the PCR.[3] The members of genus *Mycobacterium* usually have a high genomic DNA GC content and much high level of polymorphism is present among the *M. tuberculosis* species. Thorough molecular genomic studies suggest that as a species *M. tuberculosis* exhibits very little genomic sequence diversity.[11,12] Most genetic variability that has been detected is associated with transposable elements and drug resistance phenotypes.[13,14] It follows that *M. tuberculosis* should exhibit very little phenotypic variation in immunologic and virulence factors. However, evidence of phenotypic diversity among clinical isolates conflicts with this hypothesis.[15,16] The presence of significant sequence diversity in *M. tuberculosis* would provide a basis for understanding pathogenesis, immune mechanisms, and bacterial evolution. Polymorphic genes are good candidates for virulence and immune determinants, as proteins that interact directly with the host are known to have elevated divergence. Polymorphic sequences also serve as markers for phylogenetic and evolutionary studies.[15] Recently, Roche Limited has introduced an automated version of Amplicor MTB PCR referred to as the Cobas Amplicor MTB assay (F. Hoffmann-La Roche Ltd., Basel, Switzerland). In this test, the amplification, hybridization, and detection steps of the assay are accomplished with the Cobas instrument.[1] The aim of the present study is to evaluate the recently available Cobas Amplicor PCR system for the detection of MTBC in well-characterized clinical specimens and to compare the results with clinical classification and conventional culture and staining techniques. Other important goals are the assessment of the test performance for a broad collection of non-respiratory specimens and the rating of the impact on laboratory management by integrating an automated test into the workflow of a routine diagnostic laboratory.

Methods.

Study design. Three hundreds and forty-four clinical specimens, consecutively received for culture of acid-fast bacilli (AFB) by the Laboratory Department, Zayed Military Hospital, Abu Dhabi, United Arab Emirates, from 2004 to 2005, were used in this study. The specimens were collected from patients with clinical signs or symptoms of pulmonary or extrapulmonary TB or in order to exclude the possibility of TB infection.

Specimen collection and processing. The specimens investigated were collected from 344 patients (235 sputum samples, 22 bronchial leverages, 4 pus, 32 urine samples, 27 normally sterile body fluid samples, 7 biopsies, and 17 pleural body fluids). Respiratory specimens were liquefied and decontaminated by the standard N-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) method.[6] Extrapulmonary specimens, such as urine, gastric aspirates, and pleural and other similar body fluids (pericardial, synovial, and ascites fluid), were centrifuged at 3,300 x g for 15 min at 4^{O}C. The supernatant was discarded, and the pellet was resuspended in 10 ml of sterile water and decontaminated with NALC-NaOH. Part of the sediment from each specimen was inoculated onto the culture media and used for acid-fast staining, while the remainder was aliquoted and stored at -80^{O}C until the amplification techniques were performed. Cerebrospinal fluid specimens were cultured without prior decontamination.

Culture. A 0.5 ml portion of the processed sediment was cultivated with Middlebrook's and with Löwenstein-Jensen solid media. All media were incubated for 8 weeks at 36 ± 1°C. Solid media slants were inspected weekly for growth, and acid fastness from suspect colonies was confirmed by Ziehl-Neelsen's staining.

Microscopy. To detect AFB, smears were stained with auramine-rhodamine fluorescent stain and Ziel-Neelsen's stain.

Cobas Amplicor. The Cobas Amplicor test was carried out by following the instructions supplied by the manufacturer. The procedure, starting from a 100 μl sediment sample portion, consisted of 2 steps: 1) specimen preparation and 2) combined, fully automated amplification and detection. The IAC DNA sequence contained primer-binding regions identical to those of the MTB target sequence. A unique

probe-binding region differentiated the IAC from the target amplicon. The IAC was introduced into each amplification reaction and was coamplified with the possible target DNA from the clinical specimen. In addition, each run included positive and negative amplification controls. A colorimetric reading exhibiting absorbance values greater than 0.350 optical density units was considered as positive.

Statistical analysis. We used sensitivity, specificity, gold (reference) standard, positive predictive value, and negative predictive value. The culture was considered as a golden standard, and the other parameters were compared to the standard

Results. Comparison assay of the automated Cobas Amplicor MTB assay was performed for the evaluation and for ability to detect the MTB in 344 respiratory and non-respiratory clinical specimens received in our laboratory for initial diagnosis or follow-up of mycobacterial infections. The diagnostic performance of the Cobas Amplicor MTB assay was evaluated by comparison of the results of the assay with those of standard culture by using an expanded panel of culture media and acid-fast-staining methods. Out of these 344 results, 17 specimens of sputum and one pus were found to be significant and the rest were found to be non-significant. The results for all kinds of specimens are summarized in **Table 1**. There were 18 results with valuable significant information out of 344 specimens tested, 13 specimens were Cobas Amplicor MTB assay positive, and 331 specimens were Cobas Amplicor MTB assay negative. Out of these 344 specimens tested, 12 AFB stain were positive, and 332 AFB stain were negative. Of 15 culture-positive, there were 11 Cobas Amplicor MTB assay positive, which were considered as true-positive and 2 false positive appeared in the results for Cobas Amplicor MTB assay **Table 2**. Similary, out of 15 culture-positive specimens, these were 12 positive for AFB stain assay and 332 negative. Twelve specimens were considered as true positive, one false positive, 4 false negative, and 325 true negative **Table 3**. Culture assay positive were considered as true due to repeated analysis of the specimen and keeping in view the clinical history of patients and gold-standard performance of culture assay. No false-positive appeared in the results for culture assay and there were no false-negative found in culture assay. Our results are based on the previously available clinical history. Moreover, it has been suggested that our specimens were classified as true-positive, false-positive, true-negative, and false-negative, which are reconfirmed after repeated testing. In summary, the AFB stain diagnostic calculations were sensitivity = 73.3% and specificity = 99.7%. Positive predictive value = 91.7%, negative predictive value 98.8%, and the accuracy of assay was calculated as 98.5%. The final diagnostic calculation of Cobas Amplicor MTB were sensitivity = 73.3% and specificity = 99.4%. Positive predictive value = 84.6%, negative predictive value 98.8% and accuracy was 98.2% (**Table 4**).

Discussion. Molecular diagnostic techniques, such as PCR for the clinical mycobacteriology has a strong impact in terms of the timeliness and accuracy of diagnostic results. However, the promise of rapid and sensitive detection of *M. tuberculosis* directly from clinical specimens was yet remained unfulfilled, as the sensitivity of current amplification assays are lower than of culture assay.[11-13] Thus, their applications to the routine diagnosis of TB from clinical samples were remain limited. Some features considered prerequisites for screening,[14] such as partial automation and internal amplification control,

Table 1 - Results of the total 344 samples for AFP staining assay, culture assay and Cobas Amplicor *Mycobacterium tuberculosis* assay results for all kinds of specimen.

Type of specimen	Total	AFB stain positive	AFB stain negative	Culture positive	Culture negative	Cobas positive	Cobas negative
Sputum	**235**	12	223	15	220	13	222
Urine	**32**	0	32	0	32	0	32
Bronchial leverages	**22**	0	22	0	22	0	22
Pus	**4**	0	4	1	0	1	0
Pleural fluid	**17**	0	17	0	17	0	17
Sterile body fluids	**27**	0	27	0	27	0	27
Biopsies	**7**	0	7	0	7	0	7

AFB - acid fast bacilli

Table 2 - True positive, false positive, true negative and false negative results of all 3 diagnostic assays by comparison of Cobas Amplicor *Mycobacterium Tuberculosis* (MTB) assay with gold standard culture method.

Culture assay for MTB	Cobas Amplicor MTB assay	
	Cobas Amplicor total positive (N=13)	Cobas Amplicor total negative (N=331)
Total culture positive (n=15)	True positive (n=11)	False positive (n=2)
Total culture negative (n=329)	False negative (n=4)	True negative (n=325

Table 3 - Comparison of acid-fast bacilli (AFB) stain assay with gold standard culture method.

Culture assay for *Mycobacterium Tuberculosis*	AFB stain assay	
	AFB stain total positive (N=12)	AFB stain total negative (N=332)
Total culture positive (n=15)	True positive (n=11)	False positive (n=2)
Total culture negative (n=329)	False negative (n=4)	True negative (n=325)

Table 4 - Sensitivity, specificity, predictive values, and accuracy calculations for Cobas Amplicor *Mycobacterium tuberculosis* complex (MTBC) compared with culture assay results.

Diagnostic assay	Sensitivity (%)	Specificity (%)	Predictive value positive (%)	Predictive value negative (%)	Accuracy (%)
Cobas Amplicor MTB assay	73.3	99.4	84.6	98.8%	98.2
Acid-fast bacilli stain	73.3	99.7	91.7	98.8	98.5

have been implemented in the Cobas Amplicor MTB system. In addition, the automated system appears to be more sensitive and specific than its manual version.[15] By retrospective analysis of respiratory and non-respiratory specimens, the performance of the Cobas Amplicor MTB system was evaluated. The increased incidence of TB has stimulated the development of rapid assays to detect *M. tuberculosis* directly in clinical samples. We compared the 3 classical diagnostics and evaluated an in-vitro nucleic acid amplification system for the sensitive detection and specific identification of *M. tuberculosis*. In following the resolution of discrepant results, the Cobas Amplicor MTB assay percentage sensitivity was 73.3%, specificity 99.4%, positive predictive value 84.6% and negative predictive value 98.8%, which are comparable with previously in use culture assay with the accuracy of 98.2%. Cobas Amplicor MTB assay shows a rapid, specific and relatively sensitive results for the detection of MTB in clinical samples. In principle, every in-vitro nucleic acid amplification test, there were 3 typical steps to be followed: 1) nucleic acid preparation from the clinical specimen, 2) amplification of the target sequence, and 3) the specific detection of the amplicons. Although, this currently available commercial assay rely on special devices and contain commercially fabricated buffer and reagent solutions, most of the manual work has to be performed is stepwise, and the work flow is regularized by a number of necessary incubation and washing steps. With respect to the hands-on time and the laboratory's workflow, an automated device would be highly desirable. The analytical concept behind the Cobas Amplicor PCR system is based on the manual Amplicor PCR system, and both amplification and detection were fully automated. The Cobas Amplicor PCR system is the first automated nucleic acid amplification test in which the laboratory technician was able to walk away after loading the detection reagents and placing the specimens in the thermal cycler module.[3] In our study, we evaluated the performance of the Cobas Amplicor PCR system over a 2-year-period for the detection of MTB in respiratory and non-respiratory clinical specimens. We performed the diagnostic assays on every kind of specimen collected from different ethnic origins. In the course of this clinical evaluation, the diagnostic sensitivity and specificity of the Cobas Amplicor MTB assay were determined to be 73.3% for respiratory specimens and 99.4%

for non-respiratory specimens. This data were in agreement with the previously published data. Other investigators have made the same observation on respiratory specimens: sensitivities of the manual Amplicor MTB assay ranged from 66.7-86% and specificities 97-100%.[3] Therefore, assay evaluates Cobas Amplicor MTB as moderately sensitive, but highly specific. Conclusively, the 18 results obtained by the culture and Cobas Amplicor MTB assays with a good accordance are those obtained with 17 sputum and one pus specimen. While all other specimen showed reduced performance with no significant results.

In conclusion, the automated Cobas Amplicor MTB assay is well suited for use in routine clinical laboratories due to its rapid nature. Its internal control protocol can be used for monitoring inhibitory specimens. The culture assay has reached a high sensitivity and specificity, which are regarded as gold standard values, reported earlier, but Cobas Amplicor MTB assay is relatively less sensitive, equally specific but many times more rapid and less time taking compared with the culture method.The diagnostic capability of amplification assay as an adjunct to culture in the diagnosis of TB has been questioned due to the less sensitivity for same specimens. While in our assay acid-fast stain also have the same sensitivity of 73.3% and relatively similar specificity of 99.7%, which is 99.4% for Cobas Amplicor assay with a positive predictive value 91.7%, negative predictive value 98.8% and accuracy of 98.5%. Thus, in clinical practice, amplification assays have been considered relatively beneficial in the rapid differential diagnosis. Furthermore, our results suggest a potential role for the sputum and pus specimens in showing significant and evaluating results. Its specimen processing and amplification (reproducibility) and function of internal control for monitoring the presence of PCR inhibitors (reliability) are perfect which are able to provide technician, a significant reduction in hands-on time and reduction in total assay time by the truly automated amplification and detection. The role of Cobas Amplicor MTB assay for diagnosis of MTB infections is determined; it was shown to be rapid, sensitive, and specific for the detection of MTB from respiratory and non-respiratory specimens.

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