Evaluation of the COBAS AMPLICOR MTB test for the detection of *Mycobacterium tuberculosis* complex

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تقييم اختبار كوباس أمبليكور لكشف معقّد المتفطّرات السلية في نماذج سريرية

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الخلاصة: قام الباحثان بتقييم اختبار يرتكز على التفاعل السلسلي للبوليميراز باستعمال مادة كوباس أمبليكور لكشف معقّد المتفطرات السلية في عينات تنفسية وغير تنفسية عددها 866، كما أجْرَيَا التلوين الصامد للحمض، والاستنبات في مستنبت لوفنشتاين — جنسن على جميع العينات، ووجدا من بين هذه العينات جميعها 87 عينة (10.0٪) إيجابية للتفاعل السلسلي للبوليميراز ، في حين كانت 94 عينة (10.9٪) إيجابية الاستنبات، دون نتائج إيجابية كاذبة، في حين وجود 7 نتائج اعتبرت سبع عينات إيجابية الاستنبات وسلبيةً للتفاعل السلسلي للبوليميراز، سلبيةً كاذبة بعد مراجعة السجلات الطبية للمرضى. وقد لوحظ وجود معدل تثبيطي للتفاعل السلسلي للبوليميراز في 2٪ من العينات (17 عينة من بين مجموع العينات البالغ عددها 866) وذلك في العينات التنفسية وحدها. أما القيمة الإجمالية للحساسية فكانت 92.5٪، وللنوعية 100٪، أما القيمة التنبؤية الإيجابية فكانت 100٪ والقيمة التنبؤية السلبية فكانت 99.1٪. وهكذا فإن اختبار كوباس أمبليكور يُعَدُّ من الأدوات التشخيصية الهامة في المختبرات المعاصرة المتخصِّصة بعلم المتفطّرات.

ABSTRACT We evaluated the COBAS AMPLICOR polymerase chain reaction (PCR) based test for the detection of ***Mycobacterium tuberculosis*** complex in 866 respiratory and non-respiratory samples. Acid-fast staining and culture on Lowenstein–Jensen medium were also performed on all samples. Of the 866 samples tested, 87 (10.0%) were PCR-positive compared to 94 (10.9%) culture positive. There were no false positive results but 7 PCR-negative, culture-positive samples were, considered false negatives after reviewing medical records of patients. A PCR inhibitory rate of 2.0% (17/866) was observed in respiratory samples only. Sensitivity, specificity, and positive and negative predictive values for this test were 92.5%, 100%, 100% and 99.1% respectively. This test is a valuable diagnostic tool for today's mycobacteriology laboratory.

Evaluation du test COBAS AMPLICOR MTB pour la détection du complexe *Mycobacterium tuberculosis* dans des échantillons cliniques

RESUME Nous avons évalué le test COBAS AMPLICOR basé sur la PCR (amplification en chaîne par polymérase) pour détecter la présence des bactéries du complexe ***Mycobacterium tuberculosis*** dans 866 échantillons respiratoires et non respiratoires. La coloration acido-résistante et la culture sur milieu de Lowenstein-Jensen ont également été effectuées pour tous les échantillons. Sur les 866 échantillons testés, 87 (10,0 %) ont donné un résultat positif à la PCR contre 94 (10,9 %) ayant donné une culture positive. Il n'y avait pas de résultats faux positifs ; 7 échantillons négatifs à la PCR, positifs à la culture, ont toutefois été considérés comme faux négatifs après examen du dossier médical des patients. Un taux d'inhibition de la PCR de 2,0 % (17/866) a été observé dans les échantillons respiratoires seulement. Globalement la sensibilité, la spécificité et les valeurs prédictives positive et négative pour ce test s'élevaient à 92,5 %, 100 %, 100 % et 99,1 % respectivement. Le test COBAS AMPLICOR MTB est un outil diagnostique précieux pour le laboratoire de mycobactériologie de nos jours.

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Introduction

After years of decline, tuberculosis (TB) is once more becoming a worldwide problem, with 7.97 million new cases reported by the World Health Organization in 1997, of which 2 million cases proved fatal [*1*]. Direct microscopy, culture on Lowenstein–Jensen (LJ) medium and biochemical tests for detecting and identifying members of the *Mycobacterium tuberculosis* complex (MTBC) are still used in mycobacteriology laboratories. Sensitivity using microscopy is poor (of the order of 105 acid-fast bacilli/mL sputum) and culture methods require 3 to 8 weeks for completion. This merely indicates evidence of mycobacteria and additional biochemical testing is undertaken to identify the species, in itself a time consuming and challenging task necessitating experienced personnel [*2*]. The demand for sensitivity, specificity and speed of *M. tuberculosis* detection led to the development of nucleic acid-based amplification tests to target mycobacterial DNA or RNA directly from clinical samples [*3*].

Among nucleic acid-based techniques available for the diagnosis of *M. tuberculosis*, the polymerase chain reaction (PCR) is the most widely used, best studied and most widely published amplification technique. An increasing number of laboratories are using the PCR technique to detect *M. tuberculosis* in clinical samples since it provides good rates of positive results and faster turnaround times than culturing [*4*]. One of the very few commercial PCR kits available on the market currently is the COBAS AMPLICOR MTB test (Roche Diagnostic Systems Inc., Branchburg, New Jersey). This is a qualitative, in vitro, diagnostic test for the detection of MTBC in clinical samples on the COBAS AMPLICOR system. The COBAS AMPLICOR system is a semi-automated RNA and DNA amplification and detection system for routine diagnostic PCR. The system has been described in detail elsewhere [*5*].

The objective of this study was to evaluate the COBAS AMPLICOR MTB test for the routine detection of MTBC in respiratory and non-respiratory samples received at our TB laboratory. The results were compared to those obtained using conventional LJ culture medium and acid-fast staining.

Methods

Clinical samples

We carried out an open prospective study from August 1998 to May 2000. A total of 866 samples were selected from routine diagnostic respiratory and non-respiratory samples (taken from patients with clear clinical signs or symptoms of pulmonary or extra-pulmonary TB) which had been sent to the TB laboratory at the Saudi–British Laboratories for MTBC testing. The samples were collected from patients with clinical signs or symptoms of pulmonary or extra-pulmonary TB or in order to exclude the possibility of TB infection. All samples were unique, each sample represented 1 patient, and duplicate samples were excluded from this study. Respiratory samples (n = 691) analysed were: 629 sputum, 35 tracheal aspirate and 27 bronchial alveolar lavages. Non-respiratory samples (n = 175) were: 156 cerebrospinal fluid and 19 biopsies (tissues) from various sites.

Processing of samples

Respiratory samples, which are likely to contain normal or transient bacterial flora, were decontaminated. This was achieved using the N-acetyl-L-cysteine–NaOH method [*6*]. Two volumes of N-acetyl-L-cysteine–NaOH solution (4% NaOH, 1.45% Na-citrate, 0.5% N-acetyl-L-

cysteine) were mixed with the specimen on a test tube mixer for digestion. The samples were mixed until liquefied. The mixture was allowed to stand at room temperature for 15 minutes with occasional gentle shaking. Ten volumes of 6.7 mmol/L phosphate buffer (pH 7.4) were added and the mixture centrifuged at 3000 × g for 15 minutes. The resultant supernatant was decanted and the pellet washed twice in phosphate buffer. Finally, the pellet was re-suspended in 0.5 mL phosphate buffer. A 100 mL aliquot of the suspension was directly processed for PCR and the remainder inoculated onto LJ culture medium and used for acid-fast staining. For sterile samples (cerebrospinal fluid and biopsies), the decontamination process was not required and the samples were processed directly. For biopsy tissue or other tissue, usually submitted to the laboratory in a sterile Middlebrook 7H9 broth (Difco Laboratories, Detroit) to prevent dehydration, homogenization in a mechanical tissue grinder was required using the technique described by Weissfeld [*7*].

Microscopy

Fixed smears were prepared from specimen suspensions, stained with Ziehl–Neelsen (ZN) staining and examined with 100 × oil-immersion objectives using bright field microscopy. Three parallel longitudinal sweeps of the smears (i.e. approximately 100 fields per sweep, a total of 300 fields per smear) were examined according to the Centers for Disease Control recommendations [*8*].

Culture

Slants of LJ medium were inoculated with 150 mL of the prepared suspension as described in Kent and Kubica [*8*]. The slants were incubated at 37 °C for up to 8 weeks and inspected for growth twice a week.

Roche COBAS AMPLICOR MTB test

The COBAS AMPLICOR MTB test was performed on all samples according to the manufacturer's instructions. These comprise 2 steps: specimen preparation and combined amplification and detection. A detailed description of the technical procedure has been documented elsewhere [*9*]. Briefly, a 100 µL aliquot of the sample was mixed with wash solution (Tris-HCl solution containing 1% solubilizer and 0.05% sodium azide as preservative) and centrifuged (14 000 × g) for 10 minutes. After centrifugation, the supernatant was removed and lysis reagent (containing 1% solubilizer, 0.2% sodium hydroxide and 0.05% sodium azide as preservative) added to the pellet. After vortexing, the suspension was incubated at 60 °C for 45 minutes to allow for complete lysis of the mycobacterial cells. The lysed material was then neutralized by the addition of neutralizing reagent (Tris-HCl solution containing 0.05% sodium azide as preservative).

For amplification, 50 µL of the neutralized specimen was added to 50 µL of the master mix reagent. The latter contains uracil N-glycosylase, which allows safe pre-PCR enzymatic decontamination of deoxyuridine-containing PCR products, nucleotides, biotinylated primers, Taq DNA polymerase, and a synthetic internal control.

The internal control nucleic acid (DNA plasmid) contains primer-binding regions identical to those of the *M. tuberculosis* target sequence and a unique probe-binding region that differentiates the internal control from amplified mycobacterial target nucleic acid [*10*]. The internal control is introduced into each amplification reaction and co-amplified with the possible target DNA from the clinical samples. In the CO-

BAS AMPLICOR MTB test, the internal control is used at a concentration of 20 copies/test sample to indicate that amplification was sufficient to generate a positive signal from targets present at the limit of test sensitivity. The internal control is included in each sample tested to identify specimens containing substances that may interfere with PCR amplification.

During the course of this study, 1 *M. tuberculosis*-positive control and 1 *M. tuberculosis*-negative control were tested per 12-tube amplification ring. Following amplification, the instrument automatically dispenses denaturation reagent (containing 3% EDTA, 1.6% sodium hydroxide and thymol blue) to each PCR tube and a reagent containing an *M. tuberculosis*-specific oligonucleotide probe bound to paramagnetic microparticles to separate detection cups. After the addition of denatured sample or control to the detection cups, the reaction mixtures were washed 4 times and transferred to the 37 °C incubator. Colorimetric detection of the amplicons was mediated by avidin-horseradish peroxidase. An absorbance reading $A_{660} \geq 0.350$ units was considered positive for the presence of MTBC DNA. An absorbance reading $A_{660} < 0.35$ was considered negative for *M. tuberculosis*. Samples for which A_{660} is < 0.35 and internal controls for which it is ≥ 0.350 should be interpreted as negative. Samples with $A_{660} \geq 0.35$ were interpreted as positive for *M. tuberculosis* regardless of the internal control results. Samples with $A_{660} < 0.35$ and internal controls with $A_{660} < 0.35$ were interpreted as PCR-inhibitory, and the results were considered invalid.

Statistical analysis

After omitting the inhibitory samples from the statistical analysis, sensitivity, specificity, positive predictive value, and negative predictive value of the COBAS AMPLICOR MTB test were calculated by comparing the PCR results with the culture results. These calculations were based on the fact that the culture results are currently the accepted standard for TB testing [*2*]. Thus, any PCR-positive, culture-negative samples were regarded as false positive and any PCR-negative, culture-positive samples were regarded as false negative and this could only be confirmed after assessing each patient's history, symptoms, chest X-ray, tuberculin skin test result and history of drug administration, whenever those data were available.

Permission from the appropriate authority in the hospital was obtained to review the medical records for those patients.

Results

Of the 866 respiratory and non-respiratory samples tested, 49 (5.7%) were smear-positive and 817 (94.3%) were smear-negative (Table 1). All smear-positive samples were MTBC positive by the COBAS AMPLICOR MTB test. These smear-positive samples were grown successfully on LJ culture medium and isolates were identified by conventional biochemical tests as *M. tuberculosis*.

Of 866 clinical samples received, 94 (10.9%) were LJ culture positive for *M. tuberculosis*. All organisms grown on LJ slants were identified by conventional biochemical tests as *M. tuberculosis*. Of the 866 samples tested using the COBAS AMPLICOR MTB test, 87 (10.0%) were positive, 762 (88.0%) were negative and 17 (2.0%) showed PCR-inhibition.

Of the 17 inhibitory samples, 16 (94.1%) were sputum and 1 (5.9%) was bronchial alveolar lavage. All 17 samples

Table 1 **Comparison of the COBAS AMPLICOR MTB test, acid-fast bacillus (AFB) smears prepared with Ziehl–Neelsen stain and Lowenstein–Jensen (LJ) culture medium for detection of *M. tuberculosis* complex in clinical samples**

Type of specimen	No.	AFB smear		Culture (LJ medium)		COBAS AMPLICOR MTB assay		
		Positive	Negative	Positive	Negative	Positive	Negative	Inhibition[a]
Sputum	629	45	584	79	550	72	541	16
Tracheal aspirate	35	2	33	5	30	5	30	0
Bronchoalveolar lavage	27	2	25	5	22	5	21	1
Cerebrospinal fluid	156	0	156	5	151	5	151	0
Tissue (biopsy)	19	0	19	0	19	0	19	0
Total	866	49	817	94	772	87	762	17

[a]***Samples analysed with the COBAS AMPLICOR test with A_{660} < 0.35 and internal controls with A_{660} < 0.35 were interpreted as PCR-inhibitory.***

remained inhibitory despite re-testing. This inhibition was observed despite using the manufacturer's approved liquefier (N-acetyl-cysteine–NaOH) for respiratory samples.

All PCR-positive specimens were also culture-positive. There were 7 PCR-negative, culture-positive samples.

Omitting PCR-inhibitory samples from the statistical analysis and resolving discrepancies between culture and PCR results by reviewing the medical histories of the patients, the overall sensitivity, specificity, positive predictive value and negative predictive value were 92.5%, 100%, 100% and 99.1% respectively.

Discussion

The inhibition rate observed in our study (2.0%) was comparable to that reported in the literature, which ranges from 2% to 9% [*11–17*]. It is difficult to speculate on the cause of inhibition in this study, as the liquefaction method used here (N-acetyl-cysteine–NaOH) was not compared with dithiothreitol, which has previously been implicated as a cause of high inhibition rate (9.2%) [*18*]. In this study, none of the PCR-positive specimens were culture-negative.

We found no statistically significant differences in sensitivity or specificity between PCR and culture; the only difference was the accelerated detection of MTBC isolates by PCR. In comparison with the classical culture method, there were no false positive results in this study, i.e. all PCR-positive specimens were also culture-positive. There were 7 PCR-negative culture-positive samples and these were regarded as false negative, especially after reviewing the medical records for these patients, which provided clinical confirmation that they had TB. Overall sensitivity, specificity, positive predictive value and negative predictive value were consistent with previously reported values [*11–18*]. The sensitivity of this test would be lower without the built-in inhibition control. Use

of the internal control increases sensitivity because samples repeatedly found to be PCR-inhibitory were excluded from the sensitivity calculations.

Although the COBAS AMPLICOR MTB test was originally designed to detect MTBC in respiratory specimens, we have demonstrated that it can be used successfully for detecting MTBC in non-respiratory samples provided the decontamination process is omitted. Using this commercial kit, 100% sensitivity and specificity was demonstrated on cerebrospinal fluid samples. The kit will prove valuable in diagnostic clinical settings where typical growth on LJ medium and further biochemical identification takes on average 6–8 weeks compared to 5 hours using the COBAS AMPLICOR MTB test. In particular, accelerated detection of *M. tuberculosis* in cerebrospinal fluid samples will have a great impact on initiating early treatment, thereby preventing exposure of the patient to stroke, the most serious complication of TB meningitis and one which treatment with antibiotics can never rectify.

This study has demonstrated that the COBAS AMPLICOR MTB test is rapid, sensitive and highly specific. However, because of the necessity for antibiotic susceptibility testing of isolates, the high cost of using this commercial kit on every specimen arriving in the TB-laboratory and the fact that previous studies [*14,18*], in addition to our study, have demonstrated that there is no difference in sensitivity between this PCR-based test and culture methods, the COBAS AMPLICOR MTB test has not yet found widespread routine use in TB-laboratories.

Despite the above drawbacks, we believe that there is a place for this commercial PCR kit in today's TB laboratories. Each laboratory has, however, to develop its own strategy for using the kit on a select fraction of samples, and the strategy should be based on individual laboratory circumstances. In our view, the COBAS AMPLICOR MTB test can be used for (i) typing of smear-positive specimens only with potential cost savings, (ii) confirmation and exclusion of non-typical mycobacteria, saving time on biochemical identification, (iii) broth vials (radiometric detection system, BACTEC 12B broth cultures) showing growth index > 10, (iv) mycobacteria growth indicator tube (MGIT) flagged positive by the MGIT 960 system, (v) in cases where emergency testing is requested.

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