Molecular Biology Today (2002) 3: 39-42.

Potential for the Use of Polymerase Chain Reaction (PCR) in the Detection and Identification of *Mycobacterium tuberculosis* Complex in Sputum Samples

Khaled Khader Abu-Amero*

Molecular Genetics and DNA Diagnostics Laboratory, King Faisal Specialist Hospital and Research Center(MBC # 03), P.O. Box 3354, Riyadh 11211, Saudi Arabia

Abstract

The COBAS AMPLICOR *Mycobacterium tuberculosis* assay was evaluated for routine detection and identification of *Mycobacterium tuberculosis* complex (MTBC) in 629 sputum samples. In addition to the Polymerase Chain Reaction (PCR), diagnostic culture, which is considered the reference method, was also performed on theses samples. Of the 629 specimens tested, 64 were culture positive for *M. tuberculosis* and 51 were positive by COBAS AMPLICOR MTB assay and 19 specimens were considered PCR inhibitory. There were no false positive results in all tested samples and 3 PCR-negative, culture-positive specimens were considered false negative especially after reviewing the medical records for the 3 patients.

A PCR inhibition rate of 3 % (19/629) was observed; these samples remained inhibitory despite re-testing. After excluding the inhibitory samples from the statistical analysis, the overall sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) for the COBAS AMPLICOR MTB assay were 94.4, 100, 100 and 99.5 % respectively compared to those of the diagnostic culture. In conclusion, the COBAS AMPLICOR PCR system proved itself a very useful component of today mycobacteriology laboratory.

Introduction

After years of decline, tuberculosis has re-emerged as a serious public health problem worldwide. Factors contributing to this resurgence include the HIV epidemic and immigration of people from countries with a high incidence of tuberculosis. In 1993, the World Health Organization (WHO) declared it to be a global emergency and according to a recent WHO report, there were 7.96 million new cases with 2 million deaths in 1997 alone (Dye *et al*., 1999).

The mycobacteriology laboratories have to rely on conventional methods, which include direct microscopy, culture on Lowenstein-Jensen medium (LJ) and biochemical tests for detecting and identifying different members of the *M. tuberculosis* complex (MTBC). Even with concentrated samples the sensitivity of microscopy is not great (sensitivity is in the order of 10^5 acid fast bacilli per ml of sputum). Culture methods, on the other hand, are quite slow (requiring 3-8 weeks for completion). Once the presence of mycobacteria is indicated additional biochemical testing is required to identify the species. This also requires time and experienced personnel for accurate identification of isolates (Witebsky and Kruczak-Filipov, 1996).

The need for an early-accelerated detection and identification of tuberculosis, particularly in HIV patients, requires the development of diagnostic methods that have high sensitivity, specificity and rapid turnaround time of test results. The introduction of nucleic acid-based direct amplification tests to target mycobacterial DNA or RNA directly from specimens, is a most exciting milestone in diagnostic mycobacteriology and will enable detection of *M. tuberculosis* in a clinically useful time frame and with high sensitivity and specificity (Hawkey, 1994).

Among nucleic acid-based techniques, available for the diagnosis of *M. tuberculosis*, the Polymerase Chain Reaction is the most widely used; best studied and most widely published amplification technique. An increasing number of laboratories have established PCR as a supplementary test, since PCR provides good rates of positive results and better turnaround times than culture (days versus weeks) and can also identify, accurately, positive smear results (Rau and Libman, 1999).

The aim of this study was to evaluate the COBAS AMPLICOR MTB assay (Roche Diagnostic Systems, USA), an automated PCR system, for the amplification and detection of MTBC in sputum samples and compare the results obtained with the conventional LJ culture medium and the acid-fast staining technique.

Results

The COBAS AMPLICOR MTB PCR system (DiDomenico *et al*., 1996) was evaluated for its ability to detect MTBC organisms in 629 sputum samples. All samples were unique, representing a single patient each.

Of the 629 sputum samples tested, 21 (3.3 %) specimens were smear-positive and 608 (96.7 %) specimens were smear-negative. For LJ culture, 64 (10.2 %) were culture positive for *M. tuberculosis* (all organisms grown on LJ slants were identified by conventional biochemical tests as *M. tuberculosis*) (see Table-1).

The amplification results using the COBAS AMPLICOR MTB assay were as follow: of the 629 sputum samples

*For correspondence. Email kamero@kfshrc.edu.sa; Tel. (+9661) 4647272 ext. 32508; Fax. (+9661) 4427858.

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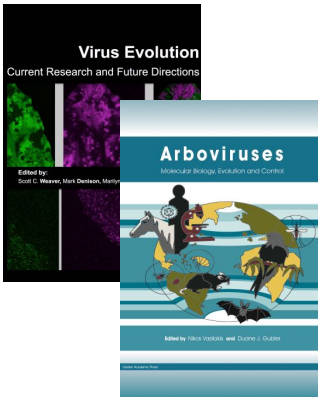

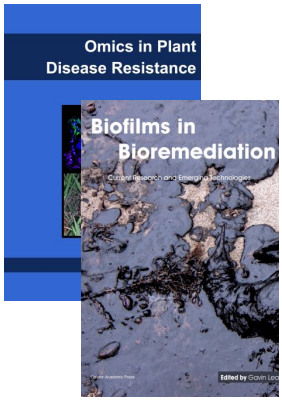

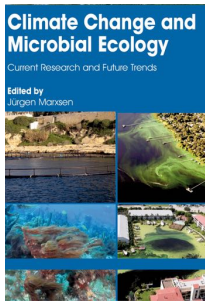

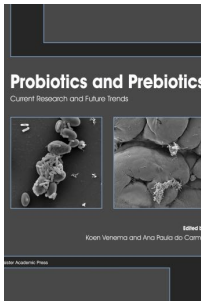

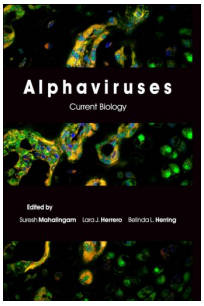

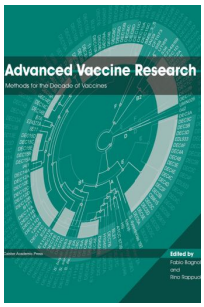

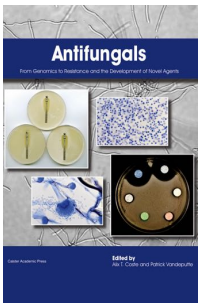

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Table 1. Comparison of the COBAS AMPLICOR MTB assay results with the ZN stain and the LJ culture media.

No. of Sputum samples (n=629) with result of						
ZN Positive	ZN Negative	Culture Positive	Culture Negative	PCR Positive	PCR Negative	PCR Inhibitory*
21 (3.3 %)	608 (96.7%)	64 (10.2%)	565 (89.8%)	51 (8.1%)	559 (88.9%)	19 (3 %)

*PCR Inhibitory, means that the PCR reading of the built in Internal Control was negative in addition to negative PCR reading of the test sample.

tested, 51 (8.1 %) were PCR positive, 559 (88.9 %) were PCR negative and 19 (3 %) were inhibitory (showing inhibition of the amplification reaction). All 19 specimens remained inhibitory despite re-testing (Table 1).

There were no false positive results in all tested specimens; all 51 PCR-positive specimens were culture-positive. However, there were 3 PCR-negative culture-positive specimens and these were regarded as false negatives, especially after reviewing patient records.

In summary, the overall positivity rate of the PCR assay were 8.1 % (51/629) compared to a positivity rate of 10.2 % (64/629) obtained using the conventional LJ culture. By omitting the 19 inhibitory specimens from the statistical calculations, the sensitivity, specificity, PPV and NPV for the COBAS AMPLICOR MTB assay were 94.4, 100, 100 and 99.5% respectively (Table 2). These calculations were based on the fact that the culture results are the current gold standard (Witebsky and Kruczak-Filipov, 1996) for TB testing. Thus, any PCR-positive, culture-negative specimens were regarded as false positive and any PCR-negative, culture-positive specimens were regarded as false negative.

Discussion

The COBAS AMPLICOR MTBC test is a qualitative *in vitro* diagnostic test for the detection of *M. tuberculosis* complex in clinical specimens on the COBAS AMPLICOR analyzer. The test utilizes the PCR nucleic acid amplification and nucleic acid hybridization techniques for the detection of *M. tuberculosis* in liquefied, decontaminated and concentrated human respiratory specimens.

Table 2. Resolved results of 629 sputum samples compared with culture

		Culture +	Culture -
COBAS AMPLICOR MTB assay results	+	51	0
	-	3	559

Sensitivity 51/54 (94.4 %)
Specificity 559/559 (100 %)
Positive predictive value 51/51 (100 %)
Negative predictive value 559/562 (99.5 %)

In this study, the COBAS AMPLICOR MTBC assay was evaluated for its capability in detecting *M. tuberculosis* in sputum samples. The results obtained using this assay were compared with results obtained with well-established gold standard techniques, culture on LJ slopes and direct microscopy for smears stained by ZN. In this study, the diagnostic sensitivity and specificity of the COBAS AMPLICOR was determined to be 94.4 and 100 % respectively. These are comparable with previously reported sensitivity and specificity rates (Jungkind *et al*., 1996; Bodmer *et al*., 1997; Reischl *et al*., 1998; Gamboa *et al*., 1998; Ninet *et al*., 1999; Wang and Tay, 1999; Katila *et al*., 2000) whereas the lowest sensitivity rate, reported in the literature, for the COBAS AMPLICOR MTBC assay was 66.3 % (Eing *et al*., 1998). In this study, the PPV and NPV were 100 and 99.5% respectively, which agrees with previously reported PPV and NPV values for the COBAS AMPLICOR MTB assay (Jungkind *et al*., 1996; Bodmer *et al*., 1997; Reischl *et al*., 1998; Gamboa *et al*., 1998; Ninet *et al*., 1999; Wang and Tay, 1999; Katila *et al*., 2000).

In comparison with classical culture methods, there were no false positive results in this study. This means that all 51 PCR-positive specimens were culture-positive. On the other hand, there were 3 PCR-negative, culture-positive specimens and these were regarded as false negative. By reviewing the records for these 3 patients, the patients have classical clinical signs of TB infection and thus these samples were regarded as confirmed PCR false negative.

COBAS AMPLICOR *M. tuberculosis* assay exhibited an inhibition rate of 3 % (19/625). This inhibition was observed despite using the manufacturer-approved liquefier (*N*-acetyl-cysteine-NaOH) for sputum samples. The inhibition rate observed here (3%) was comparable to that reported in the literature, which ranged from 2 to 9 % (Jungkind *et al*., 1996; Bodmer *et al*., 1997; Reischl *et al*., 1998; Gamboa *et al*., 1998; Ninet *et al*., 1999; Wang and Tay, 1999; Katila *et al*., 2000). It is difficult to speculate on the cause of inhibition in this study as the liquefication method used here (*N*-acetyl-cysteine-NaOH) was not compared with dithiothreitol, previously implicated as the cause of high inhibition rate of 9.2 % (Eing *et al*., 1998).

In conclusion, this study has demonstrated that the COBAS AMPLICOR MTB assay is rapid, sensitive and highly specific. I was able to process 84 sputum samples per day using this system. The system is easier to handle than 'in-house' designed PCR assays with 100 % specificity and will be a valuable tool in the clinical mycobacteriology laboratory. However, because of the necessity for susceptibility testing of isolates, it is beyond any doubt that the current amplification assays, including PCR, cannot substitute the culture method at least for now. Additionally, PCR cannot be performed on every respiratory specimen received, as the cost is prohibitive for many laboratories. As we learn more about this technology, further questions might be answered, such as how to properly evaluate the COBAS AMPLICOR MTB-positive / culture-negatives and COBAS AMPLICOR MTB – negative/ culture-positives. In addition, how the presence of PCR-inhibitors in certain samples affect the results and how to provide the clinicians with a clear picture of what exactly has been detected by

this new technology (e.g. acute infection, reactivation of the disease etc.). Despite all of this, the author firmly believes that there is a place for PCR technology in today's TB laboratories as a supplementary test (e.g. PCR could be performed on smear-positive specimens only, for confirmation and exclusion of non-typical mycobacteria, or on broth vials showing growth index > 10 in radiometric culture detection system) and that results obtained by this molecular technique should always be interpreted in conjunction with culture, microscopy and the clinical data available to the clinician.

Experimental Procedures

Patient and clinical samples

A total of 629 sputum samples were sent to the TB laboratory over the period July 1998 to March 2000. The specimens were collected from 629 patients with clinical signs and symptoms of pulmonary TB or in order to exclude the possibility of TB infection. All sputum samples were unique, each sample represents one patient, and duplicate specimens were excluded from this study.

Processing of samples

Sputum samples are likely to contain normal or transient bacterial flora and need to be decontaminated using the *N*-acetyl-L-cysteine (NALC)-NaOH method, described previously (Kubica *et al*., 1993). Two volumes of NALC-NaOH solution (4% NaOH, 1.45% Na-citrate, 0.5% NALC) were mixed with the specimen on a test tube mixer for digestion. The specimens 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 mM phosphate buffer (pH 7.4) were added and the mixture centrifuged at 3,000 x 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 of phosphate buffer. A 100 μl aliquot of the suspension was directly processed for PCR, and the remainder inoculated onto LJ culture media and used for acid fast staining.

Microscopy

Fixed smears were prepared from the specimen suspension and stained with Ziehl-Neelsen (ZN) staining (Cernoch *et al*., 1994) and examined with 100x oil-immersion objectives using bright field microscopy.

Culture

Lowenstein–Jensen (LJ) slants was inoculated with 150 μl of the prepared suspension. The slants were incubated at 37°C for up to 8 weeks and inspected for growth twice a week.

Roche COBAS AMPLICOR MTBC Assay

The Roche COBAS AMPLICOR PCR was performed according to the manufacturer's instructions. It consists of two steps: specimen preparation and combined amplification and detection. A detailed description of the technical procedure has been described previously (Jungkind *et al*., 1996). Briefly a 100 μl aliquot of the sediment sample was mixed with wash solution and centrifuged (14,000 x *g*) for 10 min. After centrifugation, the supernatant was removed and lysis reagent added to the pellet. After being vortexed, the suspension was incubated at 60°C for 45 min to allow for complete lysis of the mycobacteria. The lysed material was then neutralized by the addition of neutralizing reagent. For amplification, 50 μl of the neutralized specimen was added to 50 μl of the master mix reagent. The latter contains Uracil *N*-glycosylase enzyme, which allows safe pre-PCR enzymatic decontamination of deoxyuridine-containing PCR products, nucleotides, biotynylated primers, *Taq* DNA polymerase, and a synthetic internal control (IC).

The IC nucleic acids (DNA plasmid) contains primer-binding regions identical to those of the *M. tuberculosis* target sequence and a unique probe binding region that differentiates the IC from amplified mycobaterial target nucleic acid (Rosenstaus *et al*., 1998). The IC is introduced into each amplification reaction and co-amplified with the possible target DNA from clinical specimens. In the COBAS AMPLICOR test, the IC is used at a concentration of 20 copies per test sample to indicate that amplification was sufficient to generate a positive signal from targets present at the limit of test sensitivity. It thereby increases sensitivity by enabling the user to identify and eventually retest samples inhibitory to PCR.

During the course of this study, one *M. tuberculosis*-positive control and one *M. tuberculosis*-negative control were tested per 12-tube amplification ring. Following on-system amplification, the instrument added denaturation reagent to each PCR tube and a reagent containing an *M. tuberculosis*-specific oligonucleotide probe bound to paramagnetic micro particles to separate detection cups. After addition of denatured sample or control to the detection cups, reaction mixtures were washed four times with the manufacturer wash solution and transferred to the 37°C incubator. Colorimetric detection of the amplicons was mediated by avidin-horseradish peroxidase. An absorbance reading, A_{660} = ≥ 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*. Specimens for which A_{660} is < 0.35 and internal controls for which it is ≥ 0.350 should be interpreted as negative. Specimens with A_{660} of ≥ 0.35 are interpreted as positive for *M. tuberculosis* regardless of the internal control results. Specimens with A_{660} of < 0.35 and internal controls with A_{660} of < 0.35 should be interpreted as inhibitory, and the results should be considered invalid.

Statistical analysis

The sensitivity, specificity, positive and negative predictive values of the COBAS AMPLICOR MTB assay were calculated by contrasting the PCR results with the culture results, which were considered as the reference. Statistical comparison was performed using chi-square analysis.

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