Quality of sputum in the performance of polymerase chain reaction for diagnosis of pulmonary tuberculosis

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ABSTRACT

Setting: faster alternative techniques are required to improve the diagnosis and control of pulmonary tuberculosis. **Objective:** To evaluate the sample quality in the performance of PCR for diagnosis of pulmonary tuberculosis. **Method:** during one year, sputum samples were collected from 72 pulmonary tuberculosis patients and 12 non-tuberculosis controls, which were admitted to the Nereu Ramos hospital, Florianópolis city, Brazil. The samples were subjected to Ziehl-Neelsen-stained sputum smear microscopy and Lowestein-Jensen medium culture, which were defined as gold standard tests for mycobacteria, and polymerase chain reaction (PCR). Those samples that presented more than 40% of viable cells and less than 25% of epithelial cells were defined as high quality samples. **Results:** PCR showed sensitivity of 55.6%, specificity of 41.7%, positive predictive value of 85.1%, negative predictive value of 13.5%, and accuracy of 53.6%. High quality samples showed sensitivity of 72.4%, specificity of 50%, positive predictive value of 91.3%, negative predictive value of 20%, and accuracy of 69.7%. Low quality samples showed sensitivity of 44.2%, specificity of 37.5%, positive predictive value of 79.2%, negative predictive value of 11.1%, and accuracy of 43.1%. **Conclusion:** use of high quality samples improved significantly the PCR performance, especially on their sensitivity and positive predictive values.

Keywords: tuberculosis, sputum, polymerase chain reaction.

INTRODUCTION

Pulmonary tuberculosis, despite of all knowledge gathered on its pathogenesis, epidemiology, and therapeutics over the years, remains an important public health problem in Brazil and other developing countries. Therefore, implementation of newer diagnostic methodologies and therapeutic measures are fundamental steps for the reduction of its morbidity and mortality among the patients and for stopping its transmission chain within the community.[1]

Presently, the identification of *Mycobacterium* species relies on lengthy cultures and biochemical tests. Although molecular tests are available commercially, they are restricted to certain types of patient samples or the species of *Mycobacterium* isolated in culture.[2]

Patients with few bacilli depend strongly on the sputum culture for the definitive diagnosis, which takes on average three to six weeks to be accomplished.[3] Hence, faster alternative techniques are desirable, and among them, the polymerase chain reaction (PCR) seems to be the most promising.

PCR has popularized the use of molecular biology techniques in clinical laboratories for diagnosing many infectious diseases.[4] However, the performance of this technique depends considerably on the quality of the samples examined. Therefore, it would be of uttermost importance to know the influence of the sample quality in its performance, as well as determining criteria for acceptability of the samples in the diagnosis using this technique.

STUDY POPULATION AND METHODS

During one year, 72 pulmonary tuberculosis inpatients of hospital Nereu Ramos, Florianópolis city, Brazil, had their sputum evaluated. In addition, twelve non-tuberculosis inpatients were used as controls. The study was approved by the Human Ethic Committee of University of South of Santa Catarina, under the number 05.458.4.01.III.

The samples were collected in the morning, before breakfast, and sent as soon as possible to the laboratory. They were subjected to smear and culture for mycobacteria (both used as gold standard), and to PCR for mycobacteria.

Sputum smear

The sputum smears were stained by the Ziehl-Neelsen technique and examined according to the guidelines of the World Health Organization.[5] Briefly, heat-fixed sputum was stained with hot carbol fuchsin, decolorized with acid alcohol and then counterstained with methylene blue.

Culture of mycobacteria in Löwenstein-Jensen (LJ) medium

The samples were subjected to culture according the Petroff protocol.[5] Briefly, the samples were mixed with NaOH 4% (v/v), thoroughly shaken, and incubated for 30 minutes at 35°-37° C. Then, sterile distilled water (v/v) was added to the sample and the suspension was neutralized with 1 N HCl, containing 0.004% of phenol red. The suspension was then centrifuged at 3,000 x g for 20 minutes, and the sediment was resuspended in 500 µL of saline. A hundred microliters of the sediment was then inoculated onto two LJ medium slopes. The tubes were incubated at 35°-37° C for up to eight weeks, when the tubes with no growth were discarded as negative.

Polymerase Chain Reaction – PCR

DNA was extracted by the alkaline lysis method.[4] Briefly, the sputum was suspended in GTE (Glucose 50 mM, 10 mM EDTA, Tris/Cl PH 8.0, 0 25 mM), followed by cell lysis with SDS 1 %/NaOH 0.2 M. The suspension was neutralized with potassium acetate 3M pH 4.8-5.0. Proteinase K 20mg/mL was then added to the suspension and DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The suspension was then treated with ethanol plus sodium chloride and centrifuged. The sediment was re-suspended in 20 µL of TE (10mM Tris pH 7.4, 1 mM EDTA) and kept at -20° C until use.

The primer anti-sense MYC-264 and the primer sense F-285, which amplify a 1027 pair fragment of gene 16S rDNA from mycobacteria, were used in the PCR.[6,7] The following conditions were met: 94° C for one minute, 60° C for one minute, 35 cycles of one minute at 72° C, and one final cycle of 94° C for one minute, 60° C for one minute and 72° C for ten minutes. The amplification products were electrophoresed on a 1% (w/v) agarose gel, stained by ethidium bromide (1 µg/mL) and photographed under 320 ηm ultra-violet light (HOEFER-MacroVue UV-20), using a gel photodocumentation (DOC-PRINT® Biosystems). Their approximate size was determined by comparing it with 100 pb molecular size standard (Invitrogen).

Positive controls included DNA from Calmette-Guérin bacillus (BCG) or *Mycobacterium avium* (ATCC-25291); negative controls included DNA from *Escherichia coli* (ATCC-25922) or pure water. As additional control, a PCR amplification of AFB-negative samples was done with primers specific for eubacteria ZR-244 e F-285.[7]

Table 1. Results of PCR for high and low quality samples

| | Pulmonary TB Samples | | Control Samples | | |
| | High quality | Low quality | High quality | Low quality | Total |
	n(%)	n(%)	n(%)	n(%)	
PCR (+)	21(25)	19(22.6)	2(2.4)	5(5.9)	47(56)
PCR (-)	8(9.5)	24(28.6)	2(2.4)	3(3.6)	37(44)
Total	29(34.5)	43(51.2)	4(4.8)	8(9.5)	84(100)

Table 2. Performance of PCR for high quality and low quality samples

	Total samples	High Quality samples	Low Quality samples
Sensitivity	55.6	72.4	44.2
Specificity	41.7	50	37.5
Positive predictive value	85.1	91.3	79.2
Negative predictive value	13.5	20	11.1
Accuracy	53.6	69.7	43.1

Quality of sputum samples

High quality samples were defined as those with more than 40% of viable cells and less than 25% of epithelial cells. The viability of cells and the percentage of epithelial cells were determined according to Pizzichini *et al.* and Lee *et al.*, respectively.[8,9]

Performance of PCR

Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of PCR were calculated by the software SPSS16.0®, using the results of culture and sputum smear as gold standard.

RESULTS

Thirty-three samples (39.3%) were of high quality, while fifty-one samples (60.7%) were of low quality. PCR was positive in 47 (56%) patients and negative in 37 (44%) patients. Table 1 shows the results of the PCR in high and low quality samples, respectively.

When all samples were tested together, PCR showed sensitivity of 55.6%, specificity of 41.7%, positive predictive value of 85.1%, negative predictive value of 13.5%, and accuracy of 53.6%. In samples of high quality, PCR showed a sensitivity of 72.4%, specificity 50%, positive predictive value of 91.3%, negative predictive value of 20%, and accuracy of 69.7%. In samples of low quality, PCR showed a sensitivity of 44.2%, specificity of 37.5%, positive predictive value of 79.2%, negative predictive value of 11.1%, and accuracy of 43.1% (Table 2).

DISCUSSION

The results of this study show that the quality of the sputum influences significantly the performance of PCR for the diagnosis of pulmonary tuberculosis. The selection of samples of high quality resulted in an improved PCR, especially when the sensitivity and positive predictive value parameters where taken into account. High quality samples presented sensitivity similar to that found into another study of clinical samples, which used primers specific for the IS6110 region of *M. Tuberculosis*.[10]

Low quality sputum can represent a problem in the daily diagnosis routine. At least 25% of sputum samples sent to laboratories for culture may not be adequate, since some of them are sometimes heavily contaminated with saliva, leading to great variability in results and low reliability.[11,12]

PCR assay reduces the time for diagnosis and may increase the detection of mycobacteria in samples with negative smear results. However, variations in the procedures of in-house PCR could explain the widely variability of the sensitivity and specificity reported in several studies.[13,14,15]

The influence of respiratory tract microbiota on the performance of PCR remains disputable. According to some authors, fungi and bacteria from these sites can affect the results of PCR from sputum.[16] However, others have reported high PCR sensitivity and specificity for tuberculosis diagnosis, despite the fact that the samples were clearly contaminated with these microorganisms.[17]

In our study, we utilized only one sample for the same sputum. However, we could change this protocol, since some authors suggest that the sensitivity could be increased if the assay could be done with more samples for the same sputum.[18,19]

When testing the samples, we were not able to differentiate among different species of *Mycobacterium* because the amplified region is conserved in all species of the genus. Unlike other regions amplified, 16SrDNA expresses itself in fewer copies than the IS6110 sequences, but its amplification allows the rapid identification of subtypes by heteroduplex technique, which is in agreement with the objectives of this study.[20,21,22]

Besides the amplified region, other factors such as the amount of bacilli can influence the performance of PCR. In this regard, Wu *et al*, using nested-PRA for hsp65 gene, identified 100% of the samples with 3 + of bacilli, 95% of samples with 2 + bacilli, and only 53% of samples with 1 + or less bacilli.[23]

The fact that this study used sputum instead of culture may have contributed to lower results than those reported in the literature. However, the purpose of using sputum was an attempt to improve the technique efficiency in this type of sample by speeding up the diagnosis without the need for the culture.

CONCLUSION

Our results indicate that selection of samples of high quality resulted in an improvement in performance of PCR for the diagnosis of pulmonary TB, especially regarding its sensitivity and positive predictive value parameters.

ACKNOWLEDGMENTS

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