
SHORT COMMUNICATION

Inhibition of the Polymerase Chain Reaction by Sputum Samples from Tuberculosis Patients After Processing Using a Silica-guanidiniumthiocyanate DNA Isolation Procedure

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With the objective to evaluate PCR-mediated detection of Mycobacterium tuberculosis DNA as a diagnostic procedure for diagnosis of tuberculosis in individuals attending ambulatory services in Primary Health Units of the City Tuberculosis Program in Rio de Janeiro, Brazil, their sputum samples were collected and treated with a DNA extraction procedure using silica-guanidiniumthiocyanate. This procedure has been described to be highly efficient for extraction of different kind of nucleic acids from bacteria and clinical samples. Upon comparing PCR results with the number of acid-fast bacilli, no direct relation was observed between the number of bacilli present in the sample and PCR positivity. Part of the processed samples was therefore spiked with pure DNA of M. tuberculosis and inhibition of the PCR reaction was verified in 22 out of 36 (61%) of the samples, demonstrating that the extraction procedure as originally described should not be used for PCR analysis of sputum samples.

Key words: PCR - sputum - tuberculosis - silica-guanidiniumthiocyanate - inhibition

In 1995, 94,870 new cases of tuberculosis (TB) were reported in Brazil, being 76,840 pulmonary TB; the incidence of the disease was 58.2/100.000 (Ruffino Netto 1999). The situation is more severe in big cities like São Paulo and Rio de Janeiro, where co-infection with HIV and multi-drug resistant strains of *Mycobacterium tuberculosis* complicates efficient use of general control programs. Rapid diagnosis and adequate treatment of TB is the most efficient way to avoid spread of TB. During the last couple of years, several studies were performed to compare recently developed commercial and "in house" PCR systems with conventional diagnostic procedures such as detection of acid-fast bacilli by microscopy, culture and X-ray (Eing et al. 1998, Tortoli et al. 1999). In general, because of their high

speed, sensitivity and specificity, nucleic acid amplification systems are promising diagnostic tools; however, double blind studies have shown that the use of "in house" PCR as a routine procedure for diagnosis of TB is still controversial (Noordhoek et al. 1994, 1996, Suffys et al. 2000). More studies are needed to evaluate the sensitivity of the methods on paucibacillary material and for evaluation of feasibility and cost-effectiveness when used as a routine procedure in TB control programs, especially in developing countries (Roos et al. 1998).

One of the bottle necks of PCR for diagnostic purpose is the extraction of parasite DNA from clinical samples (Noordhoek et al. 1994). During the last decade, several methods have been published, including a very promising procedure using guanidiniumthiocyanate (GuSCN) for isolation of different types of nucleic acids from cell-rich sources and pathogenic bacteria (Boom et al. 1990). For evaluation of PCR as a diagnostic procedure of pulmonary TB in individuals attended at 14 ambulatory services in Rio de Janeiro, sputum samples were collected locally, stored at 4°C for a maximum of 4 days and further processed at the State Reference Laboratory (Central Laboratory Noel Nutels-

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Lacen, RJ). The laboratory methods for processing cultures and smears as well as for the identification of mycobacteria were standard procedures (Kent & Kubica 1985). Briefly, an aliquot of 8-10 ml of sputum was processed using NaOH-N-acetyl-L-cysteine, resuspended in 2.5 ml phosphate-buffered saline and a fraction was processed according to Boom et al. (1990), slightly modified. Briefly, 0.2 ml of the sample was added to 0.2 ml of 0.1 mm glass beads (Biospec products, Bartlesville, OK, USA) and 0.9 ml of L6 lysis buffer (Boom et al. 1990), vortexed during 10 min, submitted to two cycles of heat shock (5 min 65°C and 5 min -170°C) after which 40 µl of celite were added and the solution mixed gently at room temperature during 30 min. Supernatant was discarded and the pellet was washed twice with 1 ml of L2 buffer (Boom et al. 1990), twice with 1 ml of 70% ethanol, once with 1 ml of acetone and the pellet air dried at 56°C during 10 min. Nucleic acids were eluted in 0.1 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) during 10 min at 56°C and stored at -20°C until further use. Amplification was performed on 5 µl of the sample in a 25 µl PCR reaction using primers 5'-CCTGCGA GCGTAGGCGTCGG and 5'-TTGCTCGATCGC GTCGAGGA, flanking a 178 bp fragment of the insertion sequence IS6110 of *M. tuberculosis* (Suffys et al. 2000) and detection of the amplification product was performed on agarose gel after staining with ethidium bromide.

From the 183 sputum samples obtained from individuals with clinical and radiological diagnosis of pulmonary TB, 80 (44%) had positive PCR; when comparing PCR results with the number of bacilli observed on smears (acid fast bacilli – AFB –, +, ++ or +++), 35 (30%) of the 116 samples that were AFB –, 29 (69%) of the 42 samples that were BAAR +, 10 (77%) of 13 samples that were AFB ++ and 6 (50%) out of 12 samples that were AFB +++ were PCR positive, demonstrating that PCR positivity was not directly related to the number of bacilli present in the sample. Ninety AFB – samples had culture results: 58% was culture positive and positive PCR was observed in 48% of these, against 18% in AFB –, culture-samples. This demonstrates that *M. tuberculosis* DNA is detected more frequently in culture positive samples ($p = 0.0037$).

Because of the low PCR sensitivity in samples that had positive AFB (45 out of 67; 67%), deterioration of the parasites DNA during processing or inhibition of the PCR reaction was suspected. To verify inhibition, processed sputum samples were spiked with pure DNA of *M. tuberculosis* in the following way: purified DNA was added to the PCR mix to a final concentration of 10 ng/ml and 36 samples were submitted to PCR in the presence of this DNA; inhibition was evaluated by comparing the intensity of

the amplified product of the samples with three reactions with no clinical sample added. The three control samples had identical PCR product yield and diminished or negative PCR reaction was observed 22 samples (61%): 8 out of 17 AFB –, 4 out of 7 AFB +, 3 out of 3 AFB ++ and 7 out of 9 AFB +++, demonstrating that inhibition of the PCR reaction is occurring in a significant number of sputum samples. No attempts were made to further evaluate the influence between the volume ratio of sample and reaction mixture on PCR inhibition.

Several studies have used the GuSCN protocol for processing of clinical samples but few concentrated on sputum samples from TB patients. Although better results were obtained when comparing to other extraction protocols (Brisson-Noel et al. 1991, Noordhoek et al. 1994), these studies expressed results as PCR positivity of samples with clinically confirmed TB and did not verify inhibitors. While our study was underway, low amplification signals of cytomegalovirus (CMV) DNA in cerebrospinal fluid (CSF) and urine specimens processed with GuSCN was reported (Boom et al. 1999). Detailed analysis revealed that an inhibitor of DNA-modifying enzymes is introduced into the samples during the extraction procedures and coeluted with nucleic acids during the last step. This was not observed upon processing serum, suggesting that a silica particle-derived inhibitor can apparently be neutralized by a serum component and possibly by factors present in other tissues; addition of alpha-casein to the L6 lysis buffer prevented inhibition of PCR from CSF and urine specimens (Boom et al. 1999). Forbes and Hicks (1996) detected interference of PCR in 52% of respiratory specimens using an internal control; addition of bovine serum albumin was able to override the presence of interfering substances. We conclude from our observations that the GuSCN procedure should not be used on sputum samples without addition of internal controls or spiking experiments to verify false negative results. Sputum is one of the most frequently used sample for diagnosis of pulmonary TB and 76% of TB cases in Rio de Janeiro have the pulmonary form of the disease, demonstrating the importance of further studies to evaluate whether substances such as alpha-casein and BSA, known to enhance *Taq* polymerase activity, will improve PCR yield in sputum samples, after processing with GuSCN or other DNA extraction protocols.

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