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TECHNICAL NOTE

Evaluation of the polymerase chain reaction for the detection of *Mycobacterium tuberculosis*

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SUMMARY

The development of nucleic acid-based technologies has improved the sensitivity, specificity and speed of detection of *Mycobacterium tuberculosis* in clinical samples. Both commercially available and 'in-house' polymerase chain reaction (PCR) systems are in use, and a significant number of reports compare such systems with more traditional diagnostic tools for tuberculosis. Few studies, however, have focused on the reproducibility of the results when submitting a sample batch to PCR in different laboratories, especially in developing countries. Consequently, PCR results obtained from six laboratories in six different Latin American countries for samples reconstituted with defined amounts of *M. tuberculosis* cells were evaluated. Each laboratory used specific conditions of sample processing, nucleic acid amplification and amplicon detection. Analysis of results allowed large differences in sensitivity and specificity to be observed. We conclude that in its present setting, in-house PCR cannot be used as a single diagnostic tool for tuberculosis, and that special care needs to be taken upon interpretation of results by inclusion of a proper number of positive and negative controls.

KEY WORDS: tuberculosis; PCR; *Mycobacterium tuberculosis*

ACCORDING to the World Health Organization (WHO), 456 075 cases of tuberculosis (TB) were reported in 1996 for Latin America and the Caribbean, with over half of registered cases in Brazil, Peru and Mexico.[1] Countries such as Bolivia, Dominican Republic, Ecuador, Nicaragua, Brazil, Haiti, Honduras and Peru have an incidence of more than 50/100 000 and are considered as 'severe'; most other countries show an incidence of 25/100 000 or more.[2]

Most deaths associated with TB could have been avoided by prompt diagnosis and adequate treatment of the disease. In most countries, TB is diagnosed by detection of clinical symptoms, but these are not always specific or present in atypical forms of the disease. Diagnosis is therefore normally confirmed by chest X-ray, sputum microscopy and sample cultivation. Bacilloscopy has the disadvantage of low sensitivity and specificity, and cultures take 4 to 8 weeks, due to the slow growth of mycobacteria.

Techniques based on amplification of nucleic acids such as the polymerase chain reaction (PCR), permit detection of the infecting mycobacterial species in one working day, allowing for rapid diagnosis of TB. Several amplification procedures using different target sequences, including 'in-house' and commercial systems, have been developed, and PCR showed promising results upon evaluation as a diagnostic tool for TB.[3–11] Amplification-based procedures are still, however, technically demanding, and inter-laboratory studies on reproducibility and reliability of PCR for detection of *M. tuberculosis* are controversial.[10,11] No reproducibility studies have been performed in developing countries, but the potential of PCR as a screening procedure for TB comparing cost-effectiveness of the method versus smear examination has been reported in Kenya.[8] Also, the use of PCR for diagnosis of tuberculosis meningitis in children was recommended in the Republic of South Africa.[9] Within the framework of the Latin American and Caribbean Network on Tuberculosis (RELACTB), a study on the reproducibility of PCR was performed using results from six different countries.

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MATERIALS AND METHODS

Mycobacterium tuberculosis *culture*

The reference strain *M. tuberculosis* H37Ra (ATCC 25177) was obtained from the Instituto de Medicina Tropical Alexander von Humboldt (Lima, Peru), and grown in Middlebrook 7H9 (Difco Laboratories, Detroit, MI, USA) with 0.05% Tween 80 at 37°C. Cell growth was verified by spectrophotometry after 3 weeks, and Ziehl-Neelsen staining used for verification of culture purity. Glass beads were added to the culture for dispersion, and bacteria were counted microscopically in calibrated fields. Bacterial suspensions containing 10^3, 10^4, and 10^7 cell per ml of Middlebrook 7H9 were lyophilized until further use.

Sample preparation

Sputum was collected from individuals with respiratory manifestations different from TB, and saliva collected from healthy individuals. All samples were submitted to culture and PCR to check for the absence of *M. tuberculosis*, and stored at −20°C. Addition of *M. tuberculosis* to the samples was performed in a laboratory where no previous work with mycobacteria had been performed. The lyophilized samples were resuspended in 1 ml of deionized water, sputum, or saliva, and 100 μl fractions containing 0, 10^2, 10^3 and 10^6 bacteria, respectively, were prepared, autoclaved, and stored at −20°C. Samples were re-checked by PCR after autoclaving before being distributed among the participating laboratories. Each laboratory received the samples in triplicate and code numbered from I to VI. A set of only 30 blind samples was provided for the study, since this is the average number of samples that the laboratories test in their routine work. The laboratories used their own PCR procedures (data summarized in Table 1). The H37Ra *M. tuberculosis* strain used for preparation of samples and positive control was verified for the presence of the target sequences used in the different laboratories (data not shown).

RESULTS

None of the participants correctly identified all positive and negative *M. tuberculosis* samples. A significant number of false negatives and false positives were reported, although most false positive results were obtained for only one out of three samples (Table 2).

Reconstituted water samples

Laboratories I, II, III and IV (the later using *hsp65* as a target sequence) correctly identified the negative controls, but did not obtain positive PCR results for the samples containing 10^2 bacteria. Participant II, which applied the samples directly to the PCR reaction with no previous processing, was the only laboratory that correctly identified all negative samples, but it also gave more false negative results than any other laboratory. Only laboratory IV identified all positive samples in water using IS*6110* as a target sequence, but it obtained one false positive result using this system. A total of 21/27 (77.8%) negative

Table 1 Summary of procedures used by the participating laboratories

Laboratory*	Sample processing	PCR target (size product, bp)	PCR conditions	References	PCR analysis
I	Lysis by boiling with Triton with glass beads Phenol/chloroform extraction Ethanol precipitation	i) 32kDa protein (526bp) ii) *plc-a* (376bp) iii) 16SrDNA (208bp)	Multiprimer PCR† 30 cycles and 65°C	12 13 14	Agarose EtBr-UV
II	No	*mpt*40‡ (396bp)	PT1 and PT2 30 cycles and 65°C	15	Agarose EtBr-UV
III	Lysozyme and SDS/proteinase K lysis CTAB/chloroform extraction Isopropanol precipitation	*mpt*40‡ (396bp) IS*6110* (245bp)	PT1 and PT2 30 cycles and 60°C INS1 and INS2 30 cycles and 65°C	15 16	8% PAGE
IV	Three cycles of boiling/freezing in Triton	IS*6110* (172bp) *hsp*65 (439bp)	INS1 and FO6§ 45 cycles and 65°C Mtb1 and Mtb2 45 cycles and 60°C	16 17	Agarose EtBr-UV Southern blotting¶
V	Phenol/chloroform extraction Ethanol precipitation	IS*6110* (245bp)	INS1 and INS2 35 cycles and 65°C	16	8% PAGE
VI	Tween/proteinase K and boiling	IS*6110* (245bp)	INS1 and INS2 35 cycles and 65°C	16	Agarose EtBr-UV

* Laboratories I, IV, V and VI performed procedures such as reagent preparation, sample processing, amplification and product analysis in separate rooms.
† i) MD1 5′TCCTGACCAGCGAGCT3′ and MR1 5′CTTCATGGCGTTGAGCTG3′, amplifying a *Mycobacterium* specific fragment from the 32 kDa protein gene;[12] ii) TD1 5′CGGTCTGCGACGGCTA3′ and TR1 5′CCAGGAGACCTTGGGTA3′, amplifying a fragment from the phospholipase C gene *plc-a*, specific for *M. tuberculosis*,[13] and iii) AD3 5′GTCTCATGTTGCCAGCGG3′ and AR3 5′GTCGAGTTGCAGACCCCA3′, amplifying a fragment of the 16S rDNA gene from *M. avium-intracellulare*.[14]
‡ *mpt*40 is a fragment from the phospholipase C gene, specific for *M. tuberculosis*.[15]
§ FO6 5′TTGCTCGATCGCGTCGAGGA3′
¶ Southern blotting was performed on nylon membranes and hybridized with p32-labeled internal oligonucleotide.

Table 2 Results obtained in the detection of *M. tuberculosis* by PCR for six different laboratories

Laboratory	Volume of sample used (µl)	Sequence/primers	*M. tuberculosis* in water		*M. tuberculosis* in saliva			*M. tuberculosis* in sputum			
			0 (3)*	10^2/100 µl (3)	0 (3)	10^2/100 µl (3)	10^3/100 µl (3)	10^6/100 µl (3)	0 (3)	10^2/100 µl (3)	10^3/100 µl (3)	10^6/100 µl (3)
I	12.5	Multiprimers PCR	Negatives	Negatives	– + ++	– +d ++	+d – +	+d + +	– +d +d	Negatives	+ – –	+ – –
II	5	*mpt40*/PT1 y PT2	Negatives	Negatives	Negatives	Negatives	Negatives	– – +	Negatives	Negatives	Negatives	– + –
III	50	*mpt40*/PT1 y PT2	Negatives	Negatives	Negatives	+d +d +d	+d +d +d	+d +d +d	– +d –	Negatives	– ++ ++	Negatives
III	50	IS*6110*/INS1,INS2	Negatives	Negatives	Negatives	+d +d +d	+d + +d	+d +d ++	– + –	Negatives	– + –	– ++ ++
IV†	10	IS*6110*	– – +d	++ ++ ++	– – +	+d – –	– ++ ++	++ ++ ++	– – +	+ + –	+ – ++	++ ++ ++
IV‡	10	IS*6110*	– – +	++ ++ ++	– – +	++ – –	– ++ ++	++ ++ ++	– – +d	+d + –	++ – ++	++ + ++
IV	10	*hsp65*	Negatives	Negatives	Negatives	– – +	Negatives	+ + +	Negatives	Negatives	+ – –	+ + +
V	10	IS*6110*/INS1,INS2	– + +	– + +	+ – –	– + +	+ + –	+ – +	+ + +	+ – +	– + +	+ + +
VI	5	IS*6110*/INS1,INS2	+ + –	Negatives	– + –	+ + +	+ + –	+ + +	– + +	+ – +	– + +	+ + +
Total			21– 6+	19– 8+	21– 6+	11– 16+	11– 16+	3– 24+	16– 11+	19– 8+	14– 1 3+	8– 19+

* Values in parentheses are the number of samples tested by each laboratory.
† Detection of the 172 bp IS*6110* fragment was performed by visual inspection of 2% agarose gel stained with ethidium bromide.
‡ For detection of the 172 bp IS*6110* fragment, gels were transferred to a nylon membrane, hybridized to P32-labeled primer, and submitted to autoradiography.
+d = weakly positive; ++ = strongly positive.

samples were correctly identified by all laboratories, while only eight out of 27 (29.6%) samples containing 10^2 bacilli gave positive signals (Table 2).

Reconstituted saliva samples

Only participant III correctly identified all samples. Labs II and IV, the latter using *hsp65* as a target sequence, detected no false positive results, but detected bacilli with only low efficiency. Participants I, III, IV and VI correctly identified all samples with 10^6 bacilli. In total, 21/27 (77.8%) negative samples were PCR negative, while 16/27 (59%) samples containing 10^2 or 10^3 bacilli and 24/27 (89%) with 10^6 bacilli, were positive (Table 2).

Reconstituted sputum samples

Only participants II and IV (the latter using *hsp65*), correctly identified the negative samples, but once again a significant number of false negatives was encountered, a problem shared by the other participants. Laboratory III, where saliva samples were correctly identified, had problems in identifying sputum samples, even those with 10^6 bacilli, when using *mtp*40 as a target sequence, a problem that was partially resolved using IS*6110*. Participants IV, V, and VI (using IS*6110* as a target sequence), had identical results upon analysis of negative saliva samples (one false positive), but presented different false positives in negative sputum samples. In total, 16/27 (59%) negative samples were correctly identified, while 8/27 samples (29%) with 10^2 bacilli, 13 out of 27 (48%) with 10^3 bacilli, and 19 out of 27 (70%) with 10^6 bacilli, were positive (Table 2).

Upon analysis of all samples, the sensitivity and specificity of PCR in this experimental setting were 55% and 71.6%, respectively.

DISCUSSION AND CONCLUSIONS

The results obtained in this study demonstrate that in-house PCR is not an ideal tool for the diagnosis of tuberculosis under the present conditions in developing countries: both specificity and sensitivity are unsatisfactory. The number of organisms in saliva and sputum samples analyzed by the different laboratories ranged from a total of 5–50 bacilli to 5×10^4–5×10^5 bacilli, according to the sample volume used by each laboratory. This might be a significantly lower value than would be found in 'real' samples, and therefore the laboratories' ability to detect these organisms could be underestimated. Nevertheless, the purpose of the study was to test a wide concentration of bacilli, starting with the number of organisms detectable by PCR in sputum (1 to 100 colony forming units),[18] up to the number of acid-fast bacilli that must be present in a sample to be detectable by smear microscopy,[19] in an attempt to determine the sensitivity for sputum and saliva samples, including smear-

positive and smear-negative specimens. Our results agree with others,[18] in that sensitivity in PCR is increased in smear-positive specimens.

In general, laboratories showing high sensitivity had more problems with false positive results: participants IV and V, the only ones that obtained positive signals in water samples with 10^2 mycobacteria, also had positive PCR results for negative water samples. Most false positive results were obtained in only one out of three samples tested (Table 2). These results indicate that all samples should be made in duplicate or triplicate, and that those samples with internal discrepancy (i.e., one positive in a triplicate set) must be re-tested for confirmation. One alternative to removing false positive results would be to establish a cut-off value, but this possibility would imply a loss of true-positive results. In the present study, laboratory IV performed hybridization to increase sensitivity and specificity of PCR, and sometimes detected weak positive signals in the negative controls. This laboratory established a cut-off value, and considered four truly negative samples with slightly positive signals as negative. False positives were probably due to contamination with amplified products, since none of the laboratories that participated in this study used dUTP/uracil glycosylase (dUTP/UDG) or other decontamination systems. In addition, not all of the laboratories that participated in this study incorporated separate laboratories dedicated to each of the processes involved in clinical testing by PCR amplification: reagent preparation, specimen preparation, amplification, and product analysis (see legend, Table 1). However, the results emphasize that an accurate manipulation of the samples might be more important than the separation of working steps, as some labs using separate rooms had a high rate of cross contamination (V and VI). Special care should therefore be taken to include, besides the negative controls, a sufficient number of negative controls of sample processing, preparation of PCR mix, and sample application. In the present study, the laboratories did not use negative controls for monitoring contamination of samples during the different steps of the PCR process, and did not include positive controls to measure the efficiency of DNA extraction, amplification, and detection. In addition, no detection of PCR inhibitors in processed samples was performed in this study. However, the low sensitivity of PCR was not only due to the presence of PCR inhibitors in saliva and sputum, since several laboratories did not detect mycobacteria in water samples reconstituted with 10^2 bacilli. This could be explained by inefficient lysis and/or loss of DNA during sample preparation. Disintegration of some bacteria after autoclaving can be an explanation for the poor sensitivity of the assays, but the samples were re-checked by PCR after autoclaving before they were distributed and the results were correct. All participants had their own protocol for processing the samples, and generally different amounts of the sample were applied in the PCR reaction; this could also be partly responsible for the differences in sensitivity. In addition, two of the six participants did not use IS*6110*-PCR, nowadays accepted as one of the most sensitive systems.[20]

Similar studies on reproducibility of PCR for detection of *M. tuberculosis* have been performed,[10,11] the most recent reported in 1996 by Noordhoek et al.[10] with results obtained in 30 different laboratories from 18 countries, using both in-house and commercial PCR systems. Only five participants correctly identified all samples, seven correctly identified the positive samples and 13 the negatives. Our study complements this report, demonstrating that problems with sensitivity and specificity of PCR for detection of *M. tuberculosis* interfere with the use of the technique as a diagnostic tool, and pinpoints once more the importance of the inclusion of a sufficient number of controls, both positive and negative, in the different steps of the PCR process. The US Food and Drug Administration has published recommendations for the use of commercially available PCR systems,[21] and the American Thoracic Society organized a workshop on the evaluation of these systems for detection of *M. tuberculosis*[22] and approved the Gen-Probe MDT and the AMPLICOR *M. tuberculosis* test from Roche Diagnostic Systems Inc. According to their recommendations, the results obtained with such tests should be interpreted together with results obtained by microscopy, culture for *M. tuberculosis*, and clinical manifestations.

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RÉSUMÉ

Le développement des technologies à base d'acides nucléiques a amélioré la sensibilité et la spécificité et la vitesse de détection de *Mycobacterium tuberculosis* dans les échantillons d'origine clinique. L'on utilise des systèmes PCR d'origine commerciale ou élaborés de manière artisanale et il existe un nombre significatif de travaux comparant ces systèmes avec les outils de diagnostic plus traditionnels pour la tuberculose. Toutefois, peu d'études se sont concentrées sur la reproductibilité des résultats quand on soumet un lot d'échantillons pour PCR à différents laboratoires, en particulier dans les pays en développement. En conséquence, nous avons évalué les résultats de la PCR obtenus en provenance de six laboratoires de six pays différents d'Amérique Latine sur des échantillons reconstitués au moyen de quantités définies de *M. tuberculosis*. Chaque laboratoire a utilisé ses conditions spécifiques de traitement de l'échantillon, d'amplification de l'acide nucléique et de détection des amplicons. L'analyse des résultats a montré d'importantes différences dans la sensibilité et la spécificité. Nous concluons que dans le cadre actuel, la PCR artisanale ne peut pas être utilisée comme seul outil de diagnostic pour la tuberculose et qu'il faut porter une attention particulière à l'interprétation des résultats en incluant un nombre approprié de contrôles positifs et négatifs.

RESUMEN

El desarrollo de las tecnologías basadas en el ácido nucleico ha mejorado la sensibilidad, la especificidad y la velocidad de detección del *Mycobacterium tuberculosis* en las muestras clínicas. Están en uso los sistemas PCR comerciales y 'caseros', existe un gran número de comunicaciones que comparan estos sistemas con las armas tradicionales para el diagnóstico de la tuberculosis. Sin embargo, hay pocos estudios centrados en la reproductibilidad de los resultados cuando se envía una muestra para PCR a diferentes laboratorios, especialmente en países en desarrollo. En consecuencia, se evaluaron resultados de PCR obtenidos de seis laboratorios de seis diferentes países latino-americanos que contenían muestras reconstituídas con cantidades definidas de *M. tuberculosis*. Cada laboratorio empleó condiciones específicas de procesamiento del material, amplificación del ácido nucleico y detección del amplicon. Concluimos que en el marco actual el PCR 'casero' no puede ser utizado como un único método de diagnóstico para la tuberculosis y que deben tenerse cuidados especiales sobre la interpretación de resultados incorporando una cierta cantidad de controles positivos y negativos.