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Major Article

Single-tube nested PCR assay with in-house DNA extraction for *Mycobacterium tuberculosis* detection in blood and urine

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ABSTRACT

Introduction: Molecular analyses are auxiliary tools for detecting Koch's bacilli in clinical specimens from patients with suspected tuberculosis (TB). However, there are still no efficient diagnostic tests that combine high sensitivity and specificity and yield rapid results in the detection of TB. This study evaluated single-tube nested polymerase chain reaction (STNPCR) as a molecular diagnostic test with low risk of cross contamination for detecting *Mycobacterium tuberculosis* in clinical samples. **Methods:** *Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) was detected in blood and urine samples by STNPCR followed by agarose gel electrophoresis. In this system, reaction tubes were not opened between the two stages of PCR (simple and nested). **Results:** STNPCR demonstrated good accuracy in clinical samples with no cross contamination between microtubes. Sensitivity in blood and urine, analyzed in parallel, was 35%–62% for pulmonary and 41%–72% for extrapulmonary TB. The specificity of STNPCR was 100% in most analyses, depending on the type of clinical sample (blood or urine) and clinical form of disease (pulmonary or extrapulmonary). **Conclusions:** STNPCR was effective in detecting TB, especially the extrapulmonary form for which sensitivity was higher, and had the advantage of less invasive sample collection from patients for whom a spontaneous sputum sample was unavailable. With low risk of cross contamination, the STNPCR can be used as an adjunct to conventional methods for diagnosing TB.

Keywords: *Mycobacterium tuberculosis*. Molecular diagnostic test. Nested polymerase chain reaction. Blood. Urine.

INTRODUCTION

Tuberculosis (TB) is a major cause of morbidity and mortality affecting individuals of various ages and social classes[1]. In 2013, 83,310 cases were recorded in Brazil, including 34.7 new cases per 100,000 habitants in the Northeastern region[2] [3]. Conventional bacteriological tests for detecting TB do not combine high sensitivity and specificity[4]. For example, acid-fast bacillus (AFB) smear microscopy is rapid and low cost, but has low sensitivity and it is not specific for *Mycobacterium tuberculosis*[5].

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Molecular approaches have been used as alternative tools for diagnosing infectious diseases[5] [6] [7] [8] [9]. One of these is polymerase chain reaction (PCR), which has been applied to the detection of *M. tuberculosis*[4] [5] [7] [8] [10] [11] [12] [13] at concentrations of < 10 cells/mL in clinical samples[7] [14] [15]. Nested PCR is a variation of this technique that involves two amplification steps[7] [8] [13] [16] [17], yielding greater sensitivity and specificity[8] [16] [17]. Single-tube nested polymerase chain reaction (STNPCR) is more rapid than conventional nested PCR[8] [14] with less probability of cross-contamination and requiring a smaller amount of reagent. In STNPCR, both amplification reactions occur consecutively and the microtubes do not need to be opened or changed for the addition of new reagents[4] [8].

There are still no efficient diagnostic tests that combine high sensitivity and specificity and yield rapid results in the detection of TB[18]; this is especially challenging when few bacilli are present, for example in cases of extrapulmonary TB, pulmonary TB with a negative AFB test, childhood TB, and co-infection with TB-human immunodeficiency virus (HIV)[7] [10] [12].

Given the difficulties and delays in confirming conventional laboratory results, obtaining a timely diagnosis of TB –

especially the paucibacillary and extrapulmonary forms[10] – remains a challenge. STNPCR can achieve a more rapid diagnosis of TB than culture methods and has higher sensitivity than the AFB test using urine and blood[7] [13] [19] [20]. However, AFB, culture methods, and the GeneXpert *Mycobacterium tuberculosis* (MTB)/ rifampicin (RIF) test are still considered gold standards for diagnosing pulmonary TB in many regions[20] [21] [22] [23].

Various studies have reported the molecular diagnosis of TB using extrapulmonary samples, including in cases of negative AFB results[12] [13] [19]. The present study evaluated the performance of STNPCR in detecting the presence of *M. tuberculosis* complex in blood and urine using an in-house DNA extraction method from patients with suspected TB. The results demonstrate that the STNPCR method can improve clinical practice by providing early diagnosis of TB so as to avoid unnecessary invasive procedures and inappropriate treatment.

METHODS

Patients were non-randomly selected from public hospitals located in Recife between July 2006 and August 2009. Patients sought health services spontaneously with diverse simptons and suspected of various diseases, for example, tuberculosis or pneumonia. Sample size was determined by convenience according to the number of patients with suspected TB who sought medical attention at participating hospitals. Control patients showing pulmonary symptoms but without TB were recruited from the same hospitals.

Blood [3-5mL collected in a vacuum tube with anti-coagulant ethylenediaminetetraacetic acid (EDTA)] and urine (10ml/day for 3 consecutive days) samples were obtained from each patient suspected of TB before specific treatment was initiated. The samples were sent to Aggeu Magalhães Research Center, Oswaldo Cruz Foundation [*Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz* (CPqAM/FIOCRUZ)] within 4h of collection and stored at 4°C-8°C for up to 24h. Urine samples were decontaminated using Petroff's method[24] [25]. Blood cells were separated from whole blood at room temperature by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden).

Ethical considerations

The study protocol was approved by the Research Ethics Committee of CPqAM/FIOCRUZ (no. 0133.0.095.000-08) with the consent of participating hospitals. All participants provided written, informed consent for collection of biological samples; in cases of minors or those unable to sign or decide for themselves, this was provided by a guardian or legal representative.

Study population

Patients of both genders between the ages of 15 and 69 years and suspected of TB infection after clinical examination were recruited from public hospitals in Recife, the capital of the State of Pernambuco in Northeastern Brazil.

Patients were classified into TB and non-TB groups based on clinical, laboratory, and epidemiological criteria[22] by the physician who attended to them at the hospital, without data from molecular tests. Clinical diagnoses were made and STNPCR performed in a double-blinded fashion. Active TB was defined as a positive AFB test or TB culture according to the criteria of the Ministry of Health of Brazil[22] [26] and American Thoracic Society modified criteria[23] [27]. In cases where conventional tests were negative, inconclusive, or were not conducted, individuals were considered TB-positive if they had a clinical or radiological profile consistent with active TB and if they showed clinical improvement after treatment for 1 month with antibiotics. Latent TB was defined as a positive skin test for tuberculin and negative bacteriological exams (when it was available on patient) and with no clinical, radiological, or bacteriological evidence of active TB[23]. The negative control group consisted of individuals who used health services for reasons other than TB, who had a scar corresponding to the Bacillus Calmette-Guérin (BCG) TB vaccination, negative tuberculin test, had not had contact with a bacillipherous adult, and showed no clinical, epidemiological, or laboratory evidence compatible with TB. Patients who were human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)-positive, had chronic diseases such as kidney or heart disease, cystic fibrosis, or other chronic lung diseases, or who were using immunosuppressive drugs for more than 15 days were excluded from the study.

DNA extraction

Deoxyribonucleic acid (DNA) was extracted using a modified version of a previously published in-house protocol[28]. After DNA was bound to the Sephaglas BandPrep kit resin (Amersham Pharmacia Biotech, Little Chalfont, UK), 200µl of sodium iodide (0.9g/mL) was added to each microtube to increase bond strength. A tube without DNA served as a negative control.

Single-tube nested polymerase chain reaction

The assay was based on a previously described STNPCR method[9] and was optimized for human blood and urine. The IS6110 insert sequence (GenBank accession no. X52471) was the target for amplification. The outer primers TJ5 (5'-CCG CAA AGT GTG GCT AAC-3') and TJ3 (5'-ATC CCC TAT CCG TAT GGT G-3') amplified a 409-bp fragment, and the inner primers OLI5 (5'-AAC GGC TGA TGA CCA AAC-3') and STAN3 (5'-GTC GAG TAC GCC TTC TTG TT-3') amplified a 316-bp fragment[29]. The primers have been previously tested in blood and urine samples using a conventional nested PCR system[13].

Amplification was carried out on an automatic Eppendorf Gradient thermal cycler (Hamburg, Germany). The first stage of PCR consisted of 15 cycles (94°C for 1min, 57°C for 1 min, and 72°C for 1 min) and the second of 45 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min). For the first 15 cycles, each tube contained 0.5pmoles of outer primers in a final volume of 50µl containing 200mM Tris-HCl (pH 8.4), 500mM KCl (10× buffer), 2.5mM $MgCl_2$, 2mM dNTP (5µl), and 5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The inner primers were diluted with equal volumes of water and bromophenol blue (2µg/ml) to a final concentration

of 50 pmoles of each primer. This mixture was placed on the inner surface of the microtube cap followed by incubation at 37°C for 30 min, and then eluted from the cap surface into the reaction mixture by briefly interrupting the PCR after the 15th cycle and repeatedly inverting the tubes[9] [30]. A 10-µl volume of Tris-EDTA served as a negative control and a 2-µl volume of *M. tuberculosis* strain H37Rv DNA with 8µl Milli-Q-purified water served as a positive control for each set of reactions. PCR was repeated for all samples yielding negative results as confirmation.

Electrophoresis

Amplicons generated by STNPCR were resolved by 2% agarose gel electrophoresis. The gel was stained with ethidium bromide (20µl per liter of 1× Tris-EDTA buffer) and visualized under ultraviolet light. The Low Mass Ladder (Invitrogen) was used as a marker.

Statistical analysis

A database was created using Statistical Package for the Social Sciences (SPSS) v.10.0 software (SPSS Inc., Chicago, IL, USA) to store clinical, epidemiological, and laboratory data for all patients. PCR sensitivity and specificity and positive and negative predictive values were analyzed in parallel in leukocytes, plasma and urine[31] for each clinical sample along with their 95% confidence intervals. Note that denominated *blood sample* means leukocytes and plasma analyzed in parallel. The χ^2 test was used to evaluate the results, along with Fisher's Exact test when necessary. OpenEpi v.2.3.1 (http://www.openepi.com/oe2.3/DiagnosticTest/DiagnosticTest.htm) and SPSS v.10.0 software were used for data analysis. $P < 0.05$ was considered statistically significant.

RESULTS

We collected 167 blood and urine samples from 98 individuals (mostly male) with a mean age of 33.4 ± 18.3. A total of 83 patients were diagnosed with active or latent TB **(Table 1)** that were of two clinical forms, pulmonary and extrapulmonary. Clinical forms of extrapulmonary TB are described on **Table 2**. The performance and accuracy of STPCR were analyzed using blood and urine samples according to the clinical form of TB **(Table 3)**. When previously tested using *M. tuberculosis* strain H37Rv genomic DNA, the STNPCR system showed a detection limit of 1ag (0.001fg) (results not shown)[14].

The sensitivity of STNPCR for detecting DNA in leukocytes, plasma and urine samples from patients with or without TB was 50%, 34% and 36.5%, respectively, while the specificity was 100% relative to negative samples (**Table 4**). The p value for STNPCR on leukocytes was <0.001 and on plasma p = 0.007. The sensitivity and specificity in *blood samples* (leukocytes and plasma analyzed as one sample) were also determined[31], where a result was positive when at least one sample was positive by PCR. The sensitivity and specificity in blood were 61.5% and 100%, respectively **(Table 4)**, as compared to 65.4% and 100%, respectively, for blood and urine samples together (**Table 4**). None of patients was diagnosed with micobacteremia and only one was diagnosed as renal TB, but none had a simple genito-urinary infection.

TABLE 1 - Clinical and epidemiological characteristics of study participants.

Characteristics	Frequency	Percentage	Total
Hospital admission			
outpatients	43	43.9	-
inpatients	55	56.1	98
Sex			
male	52	53.1	-
female	46	46.9	98
Patient groups			
active TB	81	82.7	-
latent TB	2	2.0	
negative controls	15	15.3	98
Form of active TB			
pulmonary	48	59.3	-
extrapulmonary	33	40.7	81

TB: tuberculosis.

TABLE 2 - Frequency of clinical forms of extrapulmonary TB.

Clinical form of TB	Frequency	Percentage
Pulmonary	47	59.5
Pleural	17	21.5
Peripheral lymph node	3	3.8
Meningoencephalitis	1	1.3
Bone	2	2.5
Cutaneous	1	1.3
Renal	1	1.3
Other*	7	8.8
Total	**79**	**100.0**

TB: tuberculosis; *Data were not actualized in our database (missed values) for various reasons.

TABLE 3 - Determination of clinical forms of TB (pulmonary or extrapulmonary) by STNPCR analysis of blood and urine samples from individuals with or without TB.

Clinical samples	Number of samples	Pulmonary TB (95% CI)			
		sensitivity	positive predictive value	specificity	negative predictive value
Blood samples*	56	58.1% (43.3–71.6) 25/43	100.0% (77.2–100.0) 13/13	100.0% (86.7–100.0) 25/25	41.9% (26.4–59.2) 13/31
Urine	55	35.0% (22.1–50.5) 14/40	100.0% (79.6–100) 15/15	100.0% (78.5–100) 14/14	36.6% (23.6–51.9) 15/41
Blood and urine*	62	61.7% (47.4–74.2) 29/47	100.0% (79.6–100.0) 15/15	100.0% (88.3–100.0) 29/29	45.5% (29.8–62.0) 15/33
Clinical samples	**Number of samples**	**Extrapulmonary TB (95% CI)**			
		sensitivity	**positive predictive value**	**specificity**	**negative predictive value**
Blood samples*	33	65.0% (43.3–81.9) 13/20	100.0% (77.2–100.0) 13/13	100.0% (77.2–100.0) 13/13	65.0% (43.3–81.9) 13/20
Urine	47	40.6% (25.5–57.7) 13/32	100.0% (79.6–100) 15/15	100.0% (77.2–100) 13/13	44.1% (28.9–60.6) 15/34
Blood and urine*	47	71.9% (54.6–84.4) 23/32	100.0% (79.6–100) 15/15	100.0% (85.7–100) 23/23	62.5% (42.7–78.8) 15/24

TB: tuberculosis; **STNPCR:** single-tube nested polymerase chain reaction; **CI:** confidence interval. *The patient was considered positive when at least one sample was positive by STNPCR. **Note:** To determine the accuracy of STNPCR, we considered groups with pulmonary and extrapulmonary TB vs. the non-TB group (negative control + discarded TB).

TABLE 4 - Accuracy of STNPCR for clinical samples from TB patients and healthy individuals.

Clinical samples	Number of samples	STNPCR results		Predictive value	
		sensitivity	specificity	positive	negative
Leukocytes	75	50.0% (CI= 37.9–62.1) (31/62)	100.0% (CI = 77.2–100) (13/13)	100.0% (CI = 89–100) (31/31)	29.6% (CI = 18.2–44.2) (13/44)
Plasma	78	33.9% (CI = 23.5–46) (22/65)	100.0% (CI = 77.2–100) (13/13)	100.0% (CI = 85.1–100) (22/22)	23.2% (CI = 14.1–35.8) (13/56)
Blood sample (leukocytes + plasma)	78	61.5% (CI = 49.4–72.4) (40/65)	100.0% (CI = 77.2–100) (13/13)	100.0% (CI = 91.2–100) (40/40)	34.2% (CI = 21.2–50.1) (13/38)
Urine	89	36.5% (CI = 26.4–47.9) (27/74)	100.0% (CI = 79.6–100) (15/15)	100.0% (CI = 87.5–100) (27/27)	24.2% (CI = 15.3–36.2) (15/62)
Blood sample + urine	96	65.4% (CI = 54.6–74.9) (53/81)	100.0% (CI = 79.6–100) (15/15)	100.0% (CI = 93.2–100) (53/53)	34.9% (CI = 22.4–49.8) (15/43)

STNPCR: single-tube nested polymerase chain reaction; **TB:** tuberculosis; **CI:** confidence interval. **Note:** Sensitivity and specificity were calculated for non-TB (negative control + discarded TB).

DISCUSSION

A posteriori hematogenous lymphatic dissemination of Koch bacilli follows the establishment of Ghon's complex in infected patients[32], and previous studies have detected circulating bacilli in blood and urine by PCR[7][12][13]. In the present study, we evaluated the efficiency of detecting the *M. tuberculosis* complex in clinical samples by STNPCR. This method has the advantage of using samples that are easily collected with minimal invasiveness from patients with suspected pulmonary or extrapulmonary TB. This is especially useful for children. The gold standard of gastric lavage cultures requires hospitalization of the patient and is extremely invasive, and should be reserved for patients with negative AFB and extrapulmonary TB[10][12][13][14][33].

Although the STNPCR showed high sensitivity for detecting *M. tuberculosis* in blood and urine samples, is unclear why circulating bacilli were found in these samples in patients that do not have a bacteremia nor genitourinary TB[34]; the presence of *M. tuberculosis* in the blood and urine of active TB cases is typically very low, since these are paucibacillary samples. It is possible that patients with active infection have mycobacterial DNA in their macrophages and in other immune cells[35].

Recently, two automated tests for diagnosing TB directly from clinical samples have been evaluated[36], i.e., GeneXpert MTB/RIF for pulmonary TB and GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany). Both methods can detect TB in clinical samples as well as resistance to RIF and/or isoniazid[36][37][38]. Despite these advantages, the tests are only indicated for use with sputum. Therefore, a subset of TB patients with no sputum or with the extrapulmonary form of the disease cannot be diagnosed by these methods according to World Health Organization recommendations[1].

The nested PCR approach increases accuracy of detection[8][14]. In STNPCR, reactions take place in one tube for each sample, and the tubes do not need to be opened between the first and nested cycles. This decreases the risk of cross contamination between the two steps[8] and thereby increases sensitivity and specificity relative to the conventional nested PCR[13] method.

STNPCR can amplify a smaller quantity of genomic DNA than is present in a single bacillus (5fg DNA/bacillus)[14][39][40], implying that STNPCR can detect *M. tuberculosis* in samples containing only a single or even fragments of a cell[14]. It may therefore be useful in cases with no bacteriological confirmation, which would eliminate the possibility of patients receiving nonspecific treatments. Around 30% of suspected TB cases are treated in an empirical manner without bacteriological confirmation based on a set of clinical, epidemiological, and laboratory criteria or on radiological evaluation[41].

The main limitation of this study was the reference test that was used; it was neither the gold standard culture method, which has high sensitivity and specificity (10-100 bacilli/ml is considered as positive, but the test requires 3 to 8 weeks to obtain a final result[22][23]), nor the AFB test, which is rapid and low-cost but less sensitive (> 5,000 bacilli/mL is considered as positive[5][42]). Given that patients were paucibacillary, the reference test was based on an set of criteria and not one test[43]; using this approach, the sensitivity of detecting *M. tuberculosis* IS6110 in blood was

greater than that of gold standard methods[12,39], suggesting that it is reliable for confirming the presence of TB.

Tuberculosis is endemic in the Recife region[44], and without bacteriological confirmation the status of excluded TB cases is unclear. Patients classified as non-TB were diagnosed by a physician based solely on clinical suspicion[1]; TB was excluded if the patient did not respond to the treatment that was administered[1]. It is therefore possible that some excluded TB cases were falsely diagnosed as negative. On the other hand, in some cases the STNPCR was positive but these were classified as falsely positive, although some studies indicate that PCR can be considered the gold standard for infectious diseases owing to the high specificity[45,46].

The heme group of hemoglobin in whole blood acts as an inhibitor of PCR. When leukocytes and plasma were analyzed separately, this factor was eliminated[14,47,48]. In these samples, STNPCR had higher sensitivity than conventional nested PCR using whole blood[7,13,39]. The separation of blood into leukocytes and plasma as well as different primer sequences[29] may improve the results of STNPCR[7,8,13].

We analyzed the sensitivity and specificity of STNPCR for each sample regardless of the clinical form of TB **(Table 4)**. We concluded that the sensitivity of STNPCR was higher for detection of *M. tuberculosis* in plasma and leukocytes as compared to plasma alone. The sensitivity in blood samples in the present study was higher than the previously reported value[13], indicating that it is important to analyze patient plasma instead of leukocytes only.

Despite the low sensitivity of STNPCR in urine samples, when these were analyzed in parallel with blood samples, sensitivity in the latter was increased by 8%. This is consistent with another study that found that analysis of urine samples increased detection sensitivity in blood samples by 10%[12]. For this reason, the use of urine samples in PCR is recommended for TB diagnosis, especially for extrapulmonary forms or when using extrapulmonary samples[39,49]. The use of more than one clinical sample from the same patient enhances sensitivity[12] since it increases the probability of detecting DNA circulating in body fluids[10]. Another advantage of the STNPCR system is that it can be applied to blood and urine regardless of the infection site and clinical form of TB.

As for AFB and culture methods, the main difficulty in analyzing extrapulmonary TB samples by STNPCR is that they are paucibacillary[39]. PCR has been proposed as the method of choice for diagnosing TB when AFB results are negative and infection or disease is strongly suspected[40]. However, GeneXpert MTB/RIF has been suggested as being more accurate in detecting TB in smear-negative cases[50]. For positive AFB cases, PCR may still be useful in determining whether or not bacilli of the *M. tuberculosis* complex are present[38]. Nonetheless, STNPCR should only be used as an auxiliary tool for diagnosing TB since it cannot distinguish between active and latent forms of the disease[39].

In almost all cases where AFB was negative or spontaneous expectoration was not tested, STNPCR using blood and urine samples helped to establish a diagnosis of TB by detecting *M. tuberculosis*. As such, STNPCR can be useful for confirming the disease in cases that would not otherwise be diagnosed or that would be submitted to therapeutic trial. This system also has the advantages of rapidity and high specificity and sensitivity as compared to AFB and culture methods[14,51].

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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