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Standardization of a TaqMan-Based Real-Time PCR for the Detection of *Mycobacterium tuberculosis*-Complex in Human Sputum

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Abstract. Real-time polymerase chain reaction (qPCR) was optimized for detecting *Mycobacterium tuberculosis* in sputum. Sputum was collected from patients ($N = 112$) with suspected pulmonary tuberculosis, tested by smear microscopy, decontaminated, and split into equal aliquots that were cultured in Löwenstein-Jensen medium and tested by qPCR for the small mobile genetic element IS*6110*. The human *ERV3* sequence was used as an internal control. 3 of 112 (3%) qPCR failed. For the remaining 109 samples, qPCR diagnosed tuberculosis in 79 of 84 patients with culture-proven tuberculosis, and sensitivity was greater than microscopy (94% versus 76%, respectively, $P < 0.05$). The qPCR sensitivity was similar ($P = 0.9$) for smear-positive (94%, 60 of 64) and smear-negative (95%, 19 of 20) samples. The qPCR was negative for 24 of 25 of the sputa with negative microscopy and culture (diagnostic specificity 96%). The qPCR had 99.5% sensitivity and specificity for 211 quality control samples including 84 non-tuberculosis mycobacteria. The qPCR cost ~5US$ per sample and provided same-day results compared with 2–6 weeks for culture.

INTRODUCTION

Although tuberculosis (TB) diagnosis may be assumed from clinical history and radiological findings, confirmation requires laboratory-based testing. Globally, TB laboratory diagnosis relies principally on smear microscopy that has low and variable sensitivity (50–80%) and suboptimal specificity.[1,2] Culture followed by phenotypic identification and drug resistance testing is a "gold standard,"[3,4] but has the disadvantage of usually requiring several weeks and species.[5] In addition, final species identification requires additional phenotypic or genotypic testing, the latter not always being conclusive.

An alternative method for detection and/or species identification comprises the amplification of specific loci by polymerase chain reaction (PCR), which is theoretically capable of detecting a single copy of DNA from one cell. The PCR is used in the diagnosis of several infectious diseases and can give results in a few hours. For TB diagnosis, multiple commercial and in-house nucleic acid amplification techniques have been developed[6,7] and recently, a commercial real-time PCR assay has been endorsed by the World Health Organization (WHO) (Xpert RIF/TB, GeneXpert, Cepheid, Canada) for the first time.[8] However, in-house PCR assays that use equipment and reagents that are available from diverse suppliers in competitive markets may be more affordable, feasible, and sustainable than using "sole-source" tests. We therefore aimed to develop and evaluate an in-house diagnostic real-time PCR using equipment and reagents that are widely available and a non-commercial sputum processing method. We included an internal control to try to increase reliability. The performance was evaluated in patients with suspected pulmonary TB by comparing results to direct smear microscopy and culture.

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MATERIAL AND METHODS

Primer design. The primers and probe design was based on the sequence homology of the insertion sequence *6110* (IS*6110*) for all species of the *Mycobacterium tuberculosis* complex (MTBc). A 123 base pair (bp) region, which represents one of the highly conserved regions, was chosen as the target region for primer amplification. The fluorogenic BHQ-1 probe, labeled at 5′ with FAM and at 3′ with a dark quencher dye, was designed to anneal to an internal sequence of the amplified region. The sense and antisense primers and BHQ-1 probe were designated IS6110-TF, IS6110-TR, and IS6110-TP, respectively.

As an internal control to check for inhibition, a 105 bp region from *ERV3* sequence was chosen as the target for primer amplification.[9] The fluorogenic BHQ-1 probe, labeled at 5′ with HEX and at 3′ with a dark quencher dye, was designed to anneal to an internal sequence of the amplified region. The sense and antisense primers and BHQ-1 probe were designated ERV3-TF, ERV3-TR, and ERV3-TP, respectively (Table 1). All primers and probes were designed using the AlleleID v. 7.0 software (PREMIER Biosoft, Palo Alto, CA).

DNA extraction. Samples for real-time PCR (qPCR) analysis (a 10 μL loop of mycobacterial isolate or 50 μL of decontaminated sputum) were heat inactivated at 95°C for 20 min and precipitated with 300 μL cold isopropanol, incubated overnight at −20°C, centrifuged (13,000 rpm for 10 min), washed once with ice cold absolute ethanol, and the pellet was resuspended in 100 μL TE 1X (Tris 10 mM, EDTA 1 mM, pH 8.0) buffer. One microliter (1 μL) of DNA solution was used in the qPCR.

qPCR assay. The PCR was performed using a LightCycler 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany). Each multiplex PCR assay was performed in a 10 μL final reaction volume containing 2× SensiMix II Probe master (Bioline Reagents Ltd, London, UK). The following thermal profile was used: polymerase activation at 95°C for 10 min followed by 45 amplification cycles of 10 sec at 95°C and 1 min at 60°C each (annealing-extension step). A qPCR result for clinical specimens was considered negative if only a signal for *ERV3* was observed, positive in

Table 1
Primers and BHQ-probes designed and used in this study

Name	Sequence 5′→3′	Final conc. (nM)	Location (nucleotide)	Sense
IS6110-probe	TCTCAGTACACATCGATCCGGT	100	1014	+
IS6110-forward	AGACGTTATCCACCATAC	400	986	+
IS6110-reverse	AGTGCATTGTCATAGGAG	400	1108	−
ERV3-probe	CGAACCTGCACCATCAAGTCA	100	972	+
ERV3-forward	CCCAAGATAATTTCACACTAA	400	938	+
ERV3-reverse	GAGCAATACAGAATTTTCCA	400	1042	−

case of a positive IS*6110* reaction irrespective of the presence of an *ERV3* peak, and invalid if both the *ERV3* and IS*6110* reaction were negative. For qPCR the cycler threshold "C_T" is the number of PCR cycles in which the PCR product starts to be detected and represents a relative measure of the concentration of target DNA in the PCR reaction. Data were collected at the annealing step of each cycle and the C_T for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit. The IS*6110* PCR was considered positive if the C_T was ≤ 37 and the *ERV3* PCR was considered positive if the C_T was ≤ 40.

Analytical sensitivity. A pure culture of *Mycobacterium tuberculosis* reference strain Mt14323[10] was used to determine the detection limit (analytical sensitivity). Ten-fold serial dilutions from 10^7 to 1 fg (equivalent to 10^6–10° genome copies) were prepared in triplicate from the genomic DNA,[11] and each of them was mixed with 5.4 ng of human DNA derived from 1.5×10^3 peripheral blood mononuclear cells from a volunteer. The standard curve was calculated automatically by plotting the C_T values against each standard of known concentration and by extrapolating the linear regression line of this curve.

Quality control. Duplicate positive controls were included in all three of the PCR runs. We also analyzed 10 samples from the Quality Control Molecular Diagnostics 2012 *M. tuberculosis* external quality assessment panel (QCMD, Glasgow, UK) comprising eight positive controls containing *M. tuberculosis* in the concentration range 4–400 bacilli/10 μL in protein-rich buffer (0.1% Tween 20, 0.5% bovine serum albumin in phosphate buffered saline).

To assess the specificity of the qPCR all three runs included duplicate negative samples (no template control) to discriminate possible background. The external quality assessment panel also contained two negative control pooled specimens negative for MTBc. To assess the species specificity of the qPCR, we randomly selected 84 non-tuberculosis mycobacteria reference strains belonging to 35 species from the public collection of mycobacterial cultures (BCCM/ITM) (Table 2).

Clinical specimens. Single sputum samples were collected in Lima, Peru from patients ($N = 112$) with suspected TB who had been coughing for ≥ 15 days. Subjects were untreated TB patients with positive sputum smear microscopy ($N = 64$), patients with microscopy smear-negative sputum that was subsequently confirmed to be TB culture-positive ($N = 20$), and patients diagnosed as not having TB who subsequently had confirmatory negative TB smear microscopy and culture ($N = 28$).

Microbiology. An aliquot (1 mL) from each sample was decontaminated by the N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC/NaOH) method,[12] and examined by Ziehl-Neelsen staining.[13,14] A second aliquot (1 mL) of the same sample was decontaminated by the NaOH/NaCl (1:1 v/v) method,[15] the pellet resuspended in 200 μL of sterile TE 1× buffer (Tris 10 mM, EDTA 1 mM, pH 8.0), and 75 μL were inoculated onto each of two Löwenstein-Jensen tubes.

Analysis. All laboratory procedures were performed by personnel who were blinded to sample details including the results of all other tests. The clinical sensitivity and specificity of the qPCR were calculated using 2 × 2 tables by comparing results to culture on Löwenstein-Jensen medium that was considered to be the gold standard test. The 95% confidence intervals (95%CI) of proportions were calculated using the Stata program (version 12, StataCorp., College Station, TX).

Ethics. All human subjects gave informed written consent and the clinical protocols that involved sample preparation for diagnostics research were approved by the institutional ethics committee at the Universidad Peruana Cayetano Heredia

Table 2
Eighty-four samples of 35 non-tuberculosis mycobacteria strains were randomly selected to test the specificity of the assay*

Species	Biological origin	n
Mycobacterium abscessus	human	4
Mycobacterium alveum	human	1
Mycobacterium angelicum	human	1
Mycobacterium aurum		1
Mycobacterium avium	animal/human	10
Mycobacterium chelonae		1
Mycobacterium chitae		2
Mycobacterium diernhoferi		1
Mycobacterium duvalii		1
Mycobacterium engbaecki		1
Mycobacterium fallax	environment/human	2
Mycobacterium fortuitum	animal/human	8
Mycobacterium frederiksbergense	human	1
Mycobacterium genavense	animal	2
Mycobacterium gilvum	animal/human	2
Mycobacterium intracellulare	animal	6
Mycobacterium kansasii		1
Mycobacterium komossense	environment	2
Mycobacterium lentiflavum	human	1
Mycobacterium malmoense		1
Mycobacterium nonchromogenicum	environment	4
Mycobacterium obuense		1
Mycobacterium parafortuitum	environment	3
Mycobacterium peregrinum	animal/human	6
Mycobacterium rhodesiense		2
Mycobacterium scrofulaceum	environment	1
Mycobacterium septicum	human	1
Mycobacterium simiae	animal	2
Mycobacterium smegmatis		2
Mycobacterium sphagnum		2
Mycobacterium terrae		3
Mycobacterium thermoresistibile	environment	1
Mycobacterium ulcerans	human	5
Mycobacterium vaccae		1
Mycobacterium xenopi		1

*Because DNA was isolated from cultures, polymerase chain reaction (PCR) remained negative for IS*6110* and *ERV3* for all the samples except for the positive control that was run in duplicate.

and by the Regional Health Direction from the Ministry of Health. This laboratory research project used only anonymous unlinked specimens and therefore was exempted from human subjects research approval. None of the investigators have any conflict of interest in relation to this work.

RESULTS

Analytical sensitivity. Results of 10-fold serial dilutions are reported as C_T numbers versus logs of starting quantities of DNA. The PCR efficiency was 1.9 with and without the presence of human DNA (Figure 1A and B). The detection limit of this PCR assay was 10 fg of DNA/reaction which is equivalent to 1 bacillus (Figure 2).

Quality control. The results for the quality control samples are shown in Table 3. All positive control samples yielded positive qPCR results and all no template controls were negative in all qPCR runs. Among the external quality assessment panel, the qPCR results were appropriate for all 8 of 8 positive samples and 2 of 2 negative samples included as controls. All 84 non-tuberculosis mycobacteria reference strains were qPCR negative for IS*6110*. They were all negative for *ERV3* internal control, which was expected because they were isolated from cultures that did not contain human DNA. Thus, for the 106 quality control samples the sensitivity was 100% (95% CI 78–100%) and specificity was 100% (95% CI 96–100%).

Clinical specimens. All Löwenstein-Jensen cultures gave interpretable results and 100% (84 of 84) of TB cases were confirmed to be culture positive, whereas all samples from non-TB patients were culture negative. We obtained valid qPCR results in 109 of 112 samples; 3 of 112 (2.7%) others failed because they showed neither the product corresponding to the IS*6110* sequence nor the product for the *ERV3* internal control. For the valid results, qPCR analysis confirmed 94% (79 of 84) of the culture-positive samples, and predicted absence of TB DNA in 96% (24 of 25) of the negative samples. Hence, the qPCR had 94% (95% CI 90–98%)

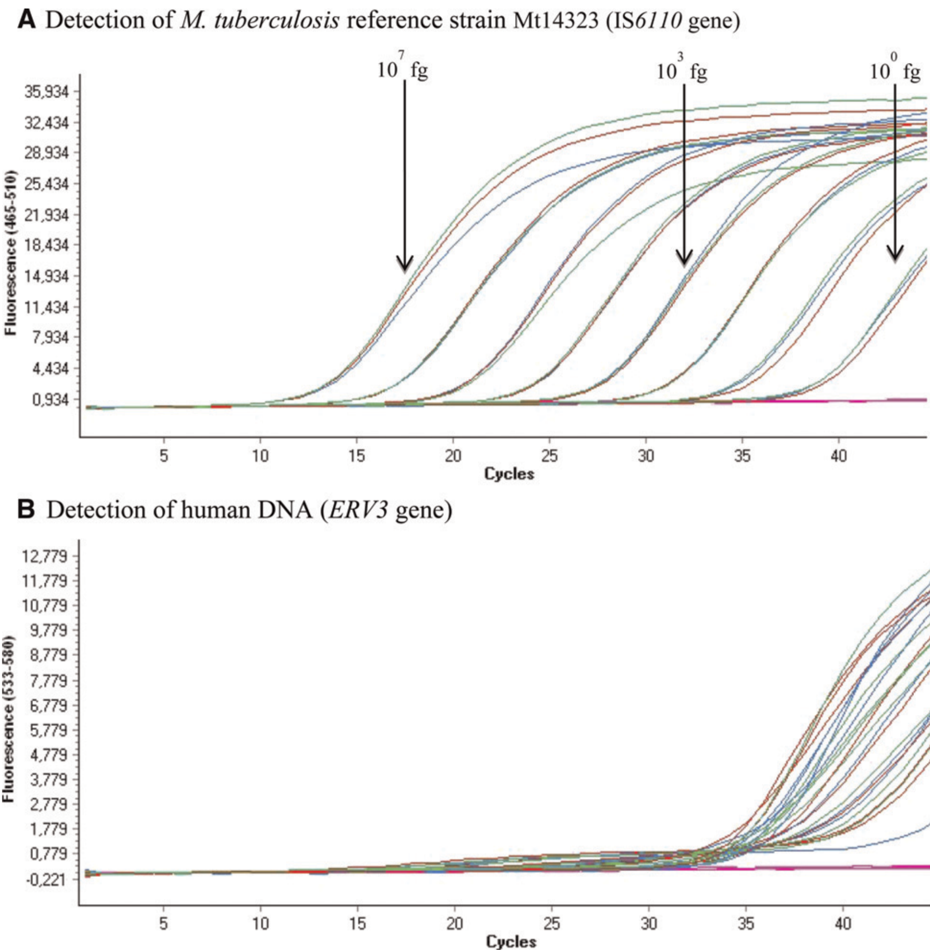

FIGURE 1. Multiplex real-time polymerase chain reaction (qPCR) assay using eight samples of which human DNA was mixed with10-fold dilutions of DNA from *Mycobacterium tuberculosis* reference strain Mt14323. Fluorescence (*y* axis) from the qPCR products is plotted against the number of cycles (*x* axis). Each experiment was run in triplicate and the results from each identical triplicate assay are shown in red, green, and blue. The flat basal purple line indicates the negative control samples. (**A**) Detection of *M. tuberculosis* IS*6110* gene with a FAM-labeled probe (465–560 nm) showing the increasing number of cycles required to detect decreasing amounts of *Mycobacterium tuberculosis* complex (MTB) DNA. The most dilute sample tested contained 10° fg, which is equivalent to the amount of DNA in a single *M. tuberculosis* bacillus and that was detected with 37 cycles. (**B**) Concurrent detection of human DNA (*ERV3*) with a VIC-labeled probe (533–580nm) in the same assays as shown in (**A**) showing that all assays were positive, indicating that these assays were valid and not prevented by the presence of PCR inhibitors. High cycle numbers were required because *ERV3* was present as a unique copy compared to IS*6110*.

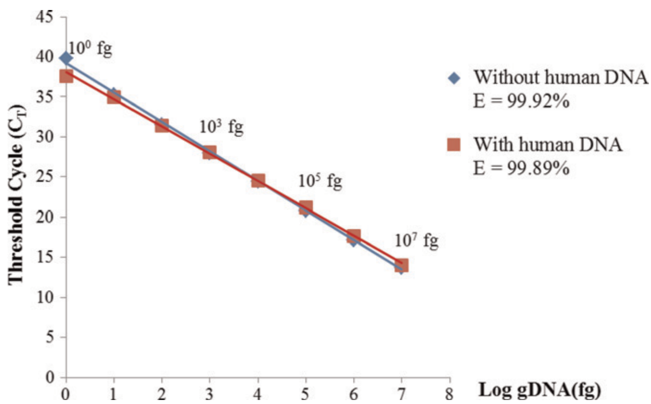

FIGURE 2. Standard curve for the real-time polymerase chain reaction qPCR analysis was done from the same stock of DNA of *Mycobacterium tuberculosis* reference strain Mt14323. Threshold cycle (*y* axis) of the reaction is plotted against the Log of *M. tuberculosis* genomic DNA (*x* axis). Threshold cycle indicates the number of cycles in which the PCR product was enough to be detected. Ten-fold serial dilutions were done from 10^7 to $10°$ fg (equivalent to 10^6–$10°$ genome copies) and were tested with (red) and without (blue) human DNA. The reaction efficiency was 99.9% for both curves.

sensitivity and 96% (95% CI 92–100%) specificity compared with Löwenstein-Jensen culture.

All quality control and clinical specimens. Considering together the 215 quality control and clinical specimens the qPCR accuracy is shown in Table 3: sensitivity 95% (95% CI 89–98% and specificity 99% (95–100%).

Speed and cost. The use of real-time PCR provided diagnostic information within 24 hours from receipt of the clinical specimen. Excluding the expense of the qPCR thermocycler device self, the running costs (reagents and materials) of this MTBc assay were ~US$5.00 for purchases in Peru.

DISCUSSION

Acid-fast bacilli (AFB) smear microscopy plays an important role in the early diagnosis of mycobacterial infections because it can be performed rapidly, within a few hours of sample collection, and requires only basic skills and equipment so it is often the only locally available diagnostic method in low-income countries. However, microscopy is prone to false negative results because it can only detect mycobacterial concentrations above ~10,000 AFB/mL of specimen. Culture is more sensitive but may be hazardous to staff and results become available only after weeks of incubation.

The development of DNA probes for rapid detection of mycobacteria has been reported, and although they may be of use in identifying cultures of *M. tuberculosis*, they lack the sensitivity required for direct detection of the organism in specimen.[16–24] Currently, several assays are commercially available for the detection of the *M. tuberculosis* complex, including the Cobas Amplicor MTB PCR test (Roche Diagnostics, Basel, Switzerland), the RealArt Mycobact Diff Kit (Qiagen, Hamburg, Germany),[25] AMTD (Gen-Probe, Inc. San Diego, CA),[26] the Inno-LiPA line probe assay (Innogenetics, Ghent, Belgium),[27] the GenoType MTDR*plus* assay (Hain Lifescience, Nehren, Germany),[28] and the Xpert RIF/TB, GeneXpert, Cepheid, Canada).[8] These commercial assays are divided into two methods: conventional PCR followed by probe hybridization and real-time PCR. They require purified DNA by enzymatic lysis and phenol-chloroform extraction, ultrasound-based platforms or commercial kits. In this study, we used heating as the only agent to break down the bacteria and to release DNA followed by ethanol precipitation, which simplified the overall procedure, making the method potentially useful for routine clinical practice.

The IS*6110* is an insertion element found exclusively within the members of the MTBc and, because of this exclusivity, it has become an important diagnostic tool in the identification of MTBc species.[29–31] McHugh and others[32] found that some non-tuberculosis mycobacteria (NTM) contain IS*6110* homologs, which potentially could affect specificity; however, in our study none of the NTM amplified. Another benefit is that this sequence has been reported to be present in multiple copies[1–25] in the genome.[33,34] Because IS*6110* can be found as several copies, our qPCR could detect as little as 0.01 pg of DNA/mL, which is equivalent

TABLE 3
Comparison of real-time PCR results with microscopy and Löwenstein-Jensen culture for detecting *Mycobacterium tuberculosis* complex

Controls/samples	*M. tuberculosis*	qPCR positive	qPCR negative	qPCR total*	Sensitivity (95% CI)	Specificity (95% CI)
Positive quality controls	Positive	14	0	14	100% (78–100%)	NA
Negative quality controls	Negative	0	8	8	NA	100% (63–100%)
Quality control non-tuberculosis mycobacteria	Negative	0	84	84	NA	100% (95–100%)
All quality control samples	Positive	14	0	102	100% (78–100%)	100% (96–100%)
	Negative	0	92			
Smear-positive patient sputum	Positive	60	4	64	94% (90–98%)	NA
Smear-negative patient sputum	Positive	19	1	20	95% (91–99%)	NA
Control patient sputum	Negative	1	24	25	NA	96% (92–100%)
All patient sputum	Positive	79	5	109	94% (90–98%)	96% (92–100%)
	Negative	1	24			
All quality control and patient sputum samples	Positive	93	5	215	95% (89–98%)	99% (95–100%)
	Negative	1	116			

*Three samples were excluded because the IS*6110* and internal control qPCR were both negative (see results).
CI = confidence intervals.

to 1 bacillus. This compares to Löwenstein-Jensen culture, which detects 10–100 viable mycobacteria/mL of sample and the Gene Xpert System's MTB/RIF assay, which detects 131 colony-forming units (CFU)/mL.[35] The most sensitive limit of detection (LOD) theoretically possible is three copies per PCR, assuming a Poisson distribution.[36] The analytical sensitivity is defined as the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure.

Five culture-positive samples had false-negative PCR results and three samples from control subjects failed because they did not show amplification for the internal control. One of the latter samples was diluted 1/10 to decrease the possible presence of inhibitors, but the result remained negative. In addition to human error, poor amplification, or the lack of enough copies of IS*6110* (< 3 copies), this could be explained by the presence of inhibitors of the amplifying system sample that may be observed in 2.9–20% of cases.[37]

This assay provides added value over previously published in-house tests by inclusion of the *ERV3* gene, an evolutionarily conserved single-copy human endogenous retrovirus with a coding envelope gene potentially involved in important placental functions.[38] The use of *ERV3* as an internal control[39] will identify the presence of false negative PCR results caused by contamination and/or inhibition.

We simplified DNA extraction to use only heat and ethanol precipitation, potentially facilitating implementation in resource-poor settings and avoiding the high cost of commercial kits for DNA isolation.[6,40] To avoid sole-source reagents and equipment that can be difficult to import, afford, and sustain in some settings, we used a TaqMan real-time PCR assay and obtained sensitivity similar to the much slower Löwenstein-Jensen culture.

Excluding the expense of the qPCR thermocycler device self, the running costs (reagents and small materials) of this MTBc assay are ~US$5.00, lower than the Xpert RIF/TB (US$10.00–$100 in different American countries) and the Hain Assay (US$10.00 in Peru).

Thus, the real-time PCR proposed in this study, showed a high level of specificity and sensitivity, short turn-around time, and relatively low cost. These results justify further evaluation of this relatively simple, low-cost test to determine diagnostic reliability in operational settings.

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