JOURNAL
OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Rapid Detection and Monitoring Therapeutic Efficacy of *Mycobacterium tuberculosis* Complex Using a Novel Real-Time Assay

Jiang, Li Juan[1†], Wen Juan Wu[2†], Hai Wu[1], Son Sik Ryang[1], Jian Zhou[2], Wei Wu[1], Tao Li[2], Jian Guo[2], Hong Hai Wang[1], Shui Hua Lu[2*], and Yao Li[1*]

[1]*State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, P.R. China*
[2]*Shanghai Public Health Clinical Center, Shanghai 201508, P.R. China*

We combined real-time RT-PCR and real-time PCR (R/P) assays using a hydrolysis probe to detect *Mycobacterium tuberculosis* complex (MTBC)-specific 16S rRNA and its rRNA gene (rDNA). The assay was applied to 28 non-respiratory and 207 respiratory specimens from 218 patients. Total nucleic acids (including RNA and DNA) were extracted from samples, and results were considered positive if the repeat RT-PCR threshold cycle was ≤35 and the ratio of real-time RT-PCR and real-time PCR load was ≥1.51. The results were compared with those from existing methods, including smear, culture, and real-time PCR. Following resolution of the discrepant results between R/P assay and culture, the overall sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of all samples (including non-respiratory and respiratory specimens) were 98.2%, 97.2%, 91.7%, and 99.4%, respectively, for R/P assay, and 83.9%, 89.9%, 72.3%, and 94.7%, respectively, for real-time PCR. Furthermore, the R/P assay of four patient samples showed a higher ratio before treatment than after several days of treatment. We conclude that the R/P assay is a rapid and accurate method for direct detection of MTBC, which can distinguish viable and nonviable MTBC, and thus may guide patient therapy and public health decisions.

Keywords: *Mycobacterium tuberculosis* complex, TaqMan PCR, therapeutic efficacy monitoring

---

*Corresponding author
Y. L.
Phone: +86-21-65642047; Fax: +86-21-65642502;
E-mail: yaoli@fudan.edu.cn
S.H. L.
Phone: +86-21-37990333-2380; Fax: +86-21-65642502;
E-mail: tubercle@shaphc.org

†These authors contributed equally to this paper.

Tuberculosis (TB) is a major global public health concern because of the high risk of person-to-person transmission, morbidity, and mortality. *Mycobacterium tuberculosis* causes nearly two million deaths and over nine million new cases of tuberculosis each year [10]. The spread of HIV/AIDS and the emergence of multidrug-resistant strains of *M. tuberculosis* are contributing to the resurgence of TB worldwide [14]. Accurate and early laboratory diagnosis is critical for effective patient management and implementation of infection control measures. Traditional laboratory smear and culture techniques are either time-consuming or lack sensitivity. Although culture is slow, it is still considered the "gold standard" for the diagnosis of TB [13].

Methods based on direct nucleic acid testing (NAT) have been developed for direct detection of the *M. tuberculosis* complex. There are currently several commercial NAT methods: the amplified *M. tuberculosis* Direct Test (MTD, Gen-Probe, San Diego, CA, USA), LCx MTB assay, ABBOTT LCx Probe System (Abbott Laboratories, Abbott Park, IL, USA), BDProbeTec ET Direct TB System (BD Diagnostics, Sparks, MD, USA), and INNO-LiPA RIF.TB assay (Innogenetics NV, Zwijndrecht, Belgium). However, although the specificity of these commercial nucleic acid amplification tests is high, the sensitivity is considerably less than that of culture [20]. In addition, these commercial nucleic acid amplification tests are often labor-intensive, requiring several hours of technical time [2, 21].

Real-time PCR techniques have revolutionized the way that clinical microbiology laboratories diagnose human pathogens, including herpes simplex virus, cytomegalovirus, *Legionella pneumophilia*, enterohemorrhagic *Escherichia coli*, hepatitis B virus, and *M. tuberculosis* [14, 17]. Real-time PCR is an improvement over conventional methods because of its increased sensitivity and specificity, low contamination risk, ease of performance, and speed [7, 24].

Several mRNA markers were introduced and published for RT-PCR-based monitoring of the efficacy of anti-TB treatment [6, 11, 12, 16, 18].

An *in vitro* study utilizing *M. smegmatis* treated with antibacterial agents concluded that detection of 16S rRNA was correlated with cell viability [25]. Because rRNA is much more abundant than mRNA, rRNA is a sensitive marker for monitoring the efficacy of anti-TB treatment.

Here, we combined real-time PCR and RT-PCR to detect MTBC-specific rDNA and its corresponding rRNA. We then assessed the performance of the assay by testing clinical specimens and comparing results with those obtained by routine microbiological techniques. Total nucleic acids (DNA and RNA) were extracted from each sample and aliquots were applied first to a real-time RT-PCR reaction, and then directly to a real-time PCR reaction. This provided the real-time RT-PCR (16S rRNA and 16S rDNA) load and the real-time PCR (16S rDNA) load, which we then used to calculate the ratio of the real-time RT-PCR load to the real-time PCR load for each sample. The assay was abbreviated to R/P in reflection of this ratio. This combined RNA/DNA assay can distinguish viable from nonviable organisms, and can therefore better identify the TB history of patients compared with a RNA marker alone. The R/P assay is also useful for monitoring patient response to therapy.

MATERIALS AND METHODS

Clinical Specimens

The study protocol was approved by the ethics committee of The Shanghai Public Health Clinical Center, Affiliated to Shanghai Fudan University. Clinical specimens (n = 235) were obtained from 218 patients with suspected tuberculosis. The types (approximate percentage) of specimens were as follows: sputum (88.1%), pleural fluid (3.0%), lymph node (3.0%), cerebrospinal fluid (2.1%), urine (1.7%), abscess and exudate (1.7%), and feces (0.4%). Clinical data were collected on standardized forms that included patient history and radiographic, laboratory, and histological findings. All specimens were tested by smear, culture, R/P assay, and real-time PCR. In those cases in which discrepant results for R/P assay and culture were obtained, the physician in charge was contacted and the clinical data of the patients were evaluated.

Processing of Specimens

Respiratory specimens were decontaminated with NaOH-*N*-acetyl-L-cysteine (NaOH-NALC), neutralized with phosphate buffer, and centrifuged at 3,000 ×g for 20 min. Extrapulmonary specimens from closed and normally sterile sites were used directly without decontamination but after a single centrifugation, as has previously been described [3].

Samples were used for the Ziehl–Neelsen stain and liquid (BACTEC MGIT 960, Becton Dickinson, USA) and solid (Lowenstein–Jensen, Becton Dickinson, USA) cultures. *Mycobacterium* isolates were identified using routine biochemical methods and commercially available molecular tests (AccuProbe, Gen-Probe, San Diego, CA, USA).

Nucleic Acid Extraction

Total nucleic acids (DNA and RNA) were isolated from homogenized sputum by a combination of organic and mechanical lysis with glass beads. In brief, 500 µl of the NaOH-NALC-treated sample was thawed and centrifuged at 13,900 ×g for 5 min. The supernatant was discarded and 45 µl of 0.05% Tween 80–TE buffer (pH 7.8) was added. The mixture was then briefly mixed on a vortex mixer. The samples were incubated at 100°C for 5 min, transferred to a 2 ml matrix tube containing 0.1 mm glass beads, and processed twice for 30 s in a MiniBeadbeater-1 (Biospec Products, Bartlesville, OK, USA). Samples were then centrifuged for 5 min at 13,900 ×g. The supernatant was purified using a D6492-01 Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. After purification, one aliquot was used for RT-PCR and a duplicate aliquot was used directly for PCR.

Oligonucleotide Primers and Probes

Full-length 16S rRNA sequences from mycobacteria isolated from humans were retrieved from the Ribosomal Database Project (http://rdp.cme.msu.edu/). The sequences were aligned using the ClustalW2 program. Primers and hybridization probes for real-time PCR were designed using Primer Express Software (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). We designed a 20 bp reverse transcription gene-specific primer (5'-CCGTATCTCAGTCCCAGTGT-3'), along with primers MTBC-16SF (5'-GGGATGCATGTCTTG TGGTG-3') and MTBC-16SR (5'-CCGTCGTCGCCTTGGTAG-3'), which amplify a 100 bp fragment of the 16S rRNA gene (GenBank Accession No. X58890). A 21 bp probe (5'-CGGGCTCATCCC ACACCGCTA-3') was synthesized (Invitrogen, Carlsbad, CA, USA) and labeled at the 5' end with 6-carboxyfluorescein (6-FAM), and with the quencher *N,N,N,N*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. A BLAST search of GenBank indicated that neither the primers nor the probes shared significant homology with other known nucleotide sequences.

Standard Sample and Standard Curve Preparation

A 16S rDNA-specific fragment was generated by conventional PCR amplification. The PCR mixture contained 400 nM of each primer, 1× buffer, 250 µM of each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Takara Bio, Otsu, Japan). Thermal cycler conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and then a final extension step of 72°C for 5 min. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced, and used as a positive control for all PCR reactions. The recombinant plasmid pGEM-T Easy-16S rRNA was quantified by UV spectroscopy. Plasmid concentrations covered the range of 10^1–10^7 copies/µl reaction at 10-fold dilutions, and an assay of each dilution was performed in triplicate. The slope of the resulting linear relation, as calculated by the ABI PRISM 7900HT operation software, was then converted into the amplification efficiency using the formula E = $10^{-1/slope}$ – 1 [15].

Reverse Transcription

Each 10 µl reaction volume contained 6 µl of extracted nucleic acid, 2 µl of 5× PrimeScript buffer, 0.25 µl of dNTP mix (10 mM each),

20 U PrimeScript Reverse Transcriptase (Takara Bio, Otsu, Japan), 100 nM gene-specific primer, and RNase-free dH$_2$O. Thermal cycling was as follows: 42°C for 30 min, 70°C for 15 min. The resulting cDNA and genomic DNA were used as a template for PCR amplification.

Real-Time PCR Assay

For real-time PCR, 0.6 µl of extracted nucleic acid (genomic DNA) was used as the template, whereas 1 µl of RT product (cDNA and genomic DNA) was used as the template for real-time RT-PCR. This insured that both reactions had an equivalent starting amount of 16S rDNA. The positive control contained 3.72 pg of plasmid DNA. The amplification mixture contained 300 nM of each primer, 400 nM fluorophore hydrolysis probe (final concentration), and 5 µl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), in a final volume of 10 µl. All reactions were optimized to obtain the best amplification kinetics under the same cycling conditions (2 min degradation of the pre-amplified templates at 50°C, 95°C for 10 min to activate *Taq* polymerase, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min).

Cut-Off Value Determination and Statistical Analysis

To determine the cut-off value, we collected 40 saliva specimens from healthy volunteers. The processing and amplification procedures for the saliva specimens were the same as for clinical specimens (see above). Using the standard curve equation, $C_t = -3.39 \times \log$ (input DNA load) + 38.38, we obtained the ratio of real-time RT-PCR (16S rRNA and 16S rDNA) load and real-time PCR (16S rDNA) load for each specimen. The cut-off value was calculated using the following formula: X + 1.96S (X indicates the mean of the ratios of 40 healthy volunteers, and S indicates the standard deviation of the ratios of 40 healthy people).

Qualitative results were obtained for each sample tested, and PCR efficiencies were calculated as previously described [4]. Assays of samples with a threshold cycle (C_t) of 35–37 were repeated. R/P assay results were considered positive if the real-time RT-PCR C_t was ≤35 and the ratio of real-time RT-PCR and real-time PCR load was ≥1.51 (cut-off value), which indicated that the clinical sample contained viable MTBC. R/P assay results were considered negative if the real-time RT-PCR C_t was >35, indicating that the clinical sample did not contain MTBC; or if the real-time RT-PCR C_t was ≤35 and the ratio of real-time RT-PCR and real-time PCR load was <1.51, which indicated that the clinical sample contained dead MTBC. Sensitivity, specificity, PPV, and NPV were calculated for all specimens submitted for *Mycobacterium* culture and R/P assay in the study. Differences between R/P assay and real-time PCR assay were determined using χ^2 tests with the aid of SPSS software (ver. 13.0). P values ≤ 0.05 were considered statistically significant.
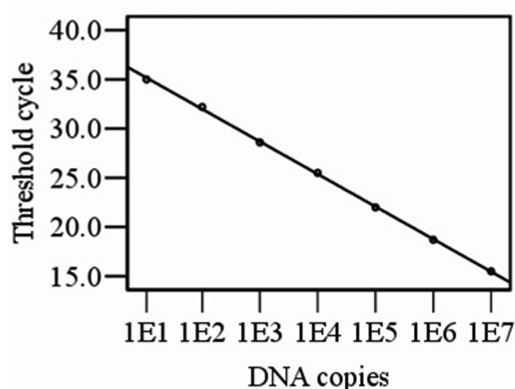

Fig. 1. Standard curve of 10-fold serial dilutions of plasmid pGEM-T Easy-16S rRNA with a correlation coefficient (R^2) of 0.999.
From the slope of the curve (s = –3.39), an amplification efficiency of E = 0.97 was calculated. The curve was generated by analysis of known amounts of template DNA using the ABI 7900 system.

RESULTS

Standard Curve Assay

A log-linear standard curve was obtained by plotting the C_t values of the assays versus the input DNA amount (Fig. 1). The limit of detection for PCR assay for the 16S rRNA fragment was 10 copies/µl, with a correlation coefficient of $R^2 = 0.999$. The amplification efficiency of real-time PCR after optimization was 0.97 when amplifying a dilution series of purified plasmid DNA containing the target region (regression curve slope, –3.39).

Clinical Sensitivity, Specificity, PPV, and NPV

Of the 235 specimens, 207 were respiratory specimens and 28 were non-respiratory specimens. Table 1 shows the source of clinical specimens and the results of culture, smear, and R/P assay.

Of the 207 respiratory specimens analyzed, 30 were culture-positive, of which 24 were smear-positive, and 177 were culture-negative, of which 27 were smear-positive. *M. tuberculosis* complex 16S rRNA and 16S rDNA were detected in all 24 smear-positive and culture-positive samples, and in four out of six smear-negative and culture-positive specimens, whereas only 21 of 177 culture-negative specimens produced positive R/P results. In total,

Table 1. Performance of the R/P assay in the diagnosis of *Mycobacterium tuberculosis* complex compared with standard culture results.

Type of specimen (no.of specimens)	Culture-positive (n)				Culture-negative(n)				Sensitivity/ Specificity	PPV/ NPV
	Smear-positive		Smear-negative		Smear-positive		Smear-negative			
	R/P+	R/P-	R/P+	R/P-	R/P+	R/P-	R/P+	R/P-		
Respiratory specimens	24	0	4	2	5	22	16	134	93.3/88.1	57.1/98.7
Non-respiratory specimens	4	0	2	0	3	2	1	16	100/81.8	60/100
All types	28	0	6	2	8	24	17	150	94.4/87.4	57.6/98.9

PPV, positive predictive values; NPV, negative predictive values.

Table 2. Results of the R/P assay compared with culture and clinical assessment of patients[a].

Type of specimen and R/P assay result	No. of cases with clinical diagnosis of TB		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Positive	Negative				
Respiratory specimens (n = 207)						
Positive	45	4	97.8	97.5	91.8	99.4
Negative	1	157				
Non-respiratory specimens (n = 28)						
Positive	10	1	100	94.4	91.0	100
Negative	0	17				
All types (n = 235)						
Positive	55	5	98.2	97.2	91.7	99.4
Negative	1	174				

[a]Values in this table are based on data post-resolution of discrepant results.
PPV, positive predictive values; NPV, negative predictive values.

there were 23 discrepant results in this group: 21 specimens were R/P-positive but negative by culture, and two specimens were R/P-negative but positive by culture (Table 1). On the basis of these data, the sensitivity, specificity, PPV, and NPV of R/P were 93.3%, 88.1%, 57.1%, and 98.7%, respectively. After a review of the patient's clinical data in those cases in which discrepant results were found, the adjusted sensitivity, specificity, PPV, and NPV of R/P were increased to 97.8%, 97.5%, 91.8%, and 99.4%, respectively (Table 2).

Of the 28 non-respiratory specimens, four were smear-positive and culture-positive, two were smear-negative and culture-positive, and 22 were culture-negative, of which five were smear-positive. *M. tuberculosis* complex 16S rRNA and 16S rDNA was detected in all four smear-positive and culture-positive samples, and in both smear-negative and culture-positive specimens. There were four discrepant results, all of which were negative by culture but positive by R/P (Table 1). On the basis of these data, the overall sensitivity of the R/P assay for non-respiratory specimens was 100%, specificity was 81.8%, PPV was 60%, and NPV was 100%. After the patient's clinical data were studied, the adjusted sensitivity, specificity, PPV, and NPV of R/P for non-respiratory specimens were determined to be 100%, 94.4%, 91.0%, and 100%, respectively (Table 2).

The overall sensitivity, specificity, PPV, and NPV of the R/P assay were 94.4%, 87.4%, 57.6%, and 98.9%

respectively, when the culture results were used as reference standard, and 98.2%, 97.2%, 91.7%, and 99.4%, respectively, when the discrepant results were resolved.

Comparison of the R/P Assay and Real-Time PCR Assay

Of the 235 clinical specimens analyzed, the R/P assay showed a higher sensitivity (98.2% versus 83.9%, p < 0.05) and a higher specificity (97.2% versus 89.9%, p < 0.05) than the PCR assay, when the discrepant results were resolved (Table 3). The PPV and NPV of the R/P assay were 91.7% and 99.4%, respectively, which was significantly better than the PPV and NPV of the PCR assay (91.7% versus 72.3%, p < 0.05; 99.4% versus 94.7%, p < 0.05) (Table 3).

Monitoring Therapeutic Effects

Of the total 218 patients with suspected tuberculosis, four positive patients were followed up with second or third specimens (n ≥ 2). The progress of antimicrobial therapy in these patients could be monitored using the R/P assay in parallel with culture and clinical assessment, but the real-time PCR results from different stages of the treatment were always positive. At the initiation of antimicrobial therapy, the R/P assay for the four patients showed a higher ratio than after several days of treatment (Table 4).

Table 3. Performance of PCR and R/P assays with hydrolysis probes for detecting *Mycobacterium tuberculosis* complex 16S rRNA and 16S rDNA in clinical samples, compared with culture and clinical assessment of patients[a].

Real-time assay	True positive (n)	False negative (n)	False positive (n)	True negative (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
PCR	47	9	18	161	83.9	89.9	72.3	94.7
R/P	55	1	5	174	98.2*	97.2*	91.7*	99.4*

[a]Values in this table are based on data post-resolution of discrepant results.
*Significant difference, χ^2 tests (p < 0.05), as compared with real-time PCR assay.
PPV, positive predictive values; NPV, negative predictive values.

Table 4. TB-diagnosed patients whose treatment was monitored using culture, smear, PCR, and R/P assay.

	Therapy (days)	Culture	Smear	Real-time PCR	R/P	R/P ratio
Patient 1	0	+	+	+	+	6.2
	35	+	+	+	+	3.3
	75	−	−	+	−	1.2
Patient 2	0	+	+	+	+	4.0
	20	+	+	+	+	2.5
	50	−	+	+	−	1.4
Patient 3	0	+	+	+	+	4.5
	63	−	−	+	−	0.9
Patient 4	0	+	+	+	+	3.8
	40	−	+	+	−	1.2

+, Positive; −, negative.

DISCUSSION

Molecular techniques have had considerable impact on the rapid diagnosis and clinical monitoring of MTBC infection. Numerous rapid diagnostic tests employing a number of different MTBC amplification targets, including 16S rRNA [8, 20, 21, 25] and 16S rDNA [5, 19, 23], have been described previously. We developed a novel approach in which 16S rRNA- and 16S rDNA-based real-time PCR assays were combined to detect MTBC in clinical samples. This combined approach offers several major advantages. Because the same DNA fragment is being amplified, with the same amplification efficiency, the ratio is more reliable for distinguishing viable and nonviable MTBC. This is beneficial for fast patient management and assessment of patient response to standard therapy. In addition, the R/P assay can assess the TB history of patients, and is extremely fast, sensitive, and specific, as demonstrated here.

The overall sensitivity and specificity of the R/P assay for all sample types was higher when the discrepant results were resolved, as compared with that obtained when cultures were used as a reference standard. It is generally accepted that the sensitivity of culture methods for the diagnosis of TB is not ideal, particularly for paucibacillary forms of the disease, and positive cases may be missed by cultures alone [1].

Table 3 summarizes a comparison between the R/P assay and the PCR assay. The sensitivity and specificity of the R/P assay was significantly better than the PCR assay in all cases. The copy number of rRNA is much higher than that of DNA in the template, so real-time RT-PCR had greater sensitivity than real-time PCR. Because clinical samples with dead MTBC were also considered negative for the R/P assay, the R/P assay demonstrated higher specificity than the real-time PCR assay.

In our study, the data obtained from TB-diagnosed patients whose treatment was monitored using culture, smear, PCR, and R/P assay indicated that decay of 16S rRNA occurs rapidly after cell death [9], unlike degradation of DNA. This is consistent with previous reports, which have found positive PCR signals in both viable and dead cells as long as intact DNA was present [22, 26]. Table 4 summarizes the relationship between R/P ratio and days of therapy for patients. As expected, we observed that the ratio of R/P decreased along with the days of treatment. This may suggest that it is possible to use the R/P assay as a final "test of cure" to monitor therapy efficacy. One limitation of our study is that the patient number was small. We plan to continue our study for the monitoring of the efficacy of anti-TB treatment in the future.

In conclusion, the R/P assay is a rapid and specific method for direct detection of MTBC in clinical specimens. The high level of automation and containment helped avoid contamination. The R/P assay can distinguish viable and nonviable MTBC, and can better guide patient therapy and public health decisions by combining with culture and smear results [18].

Acknowledgments

REFERENCES

1. Al Zahrani, K., H. Al Jahdali, L. Poirier, P. Rene, M. Gennaro, and D. Menzies. 2000. Accuracy and utility of commercially available amplification and serologic tests for the diagnosis of minimal pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* **162:** 1323–1329.

2. Araj, G., R. Talhouk, L. Itani, W. Jaber, and G. Jamaleddine. 2000. Comparative performance of PCR-based assay versus microscopy and culture for the direct detection of

Mycobacterium tuberculosis in clinical respiratory specimens in Lebanon. *Int. J. Tuberc. Lung Dis.* **4:** 877−881.

3. Broccolo, F., P. Scarpellini, G. Locatelli, A. Zingale, A. M. Brambilla, P. Cichero, *et al.* 2003. Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. *J. Clin. Microbiol.* **41:** 4565−4572.

4. Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, *et al.* 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55:** 611−622.

5. Cloud, J., H. Neal, R. Rosenberry, C. Turenne, M. Jama, D. Hillyard, *et al.* 2002. Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. *J. Clin. Microbiol.* **40:** 400−406.

6. DesJardin, L. E., M. D. Perkins, K. Wolski, S. Haun, L. Teixeira, Y. Chen, *et al.* 1999. Measurement of sputum *Mycobacterium tuberculosis* messenger RNA as a surrogate for response to chemotherapy. *Am. J. Respir. Crit. Care Med.* **160:** 203−210.

7. Espy, M., J. Uhl, L. Sloan, S. Buckwalter, M. Jones, E. Vetter, *et al.* 2006. Real-time PCR in clinical microbiology: Applications for routine laboratory testing. *Clin. Microbiol. Rev.* **19:** 165−256.

8. Foongladda, S., S. Pholwat, B. Eampokalap, P. Kiratisin, and R. Sutthent. 2009. Multi-probe real-time PCR identification of common *Mycobacterium* species in blood culture broth. *J. Mol. Diagn.* **11:** 42−48.

9. Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K. H. Schleifer. 1992. Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. *J. Gen. Microbiol.* **138:** 879−887.

10. Harries, A. D. and C. Dye. 2006. Tuberculosis. *Ann. Trop. Med. Parasitol.* **100:** 415−431.

11. Hellyer, T., L. DesJardin, G. Hehman, M. Cave, and K. Eisenach. 1999. Quantitative analysis of mRNA as a marker for viability of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **37:** 290−295.

12. Hellyer, T., L. DesJardin, L. Teixeira, M. Perkins, M. Cave, and K. Eisenach. 1999. Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA. *J. Clin. Microbiol.* **37:** 518−523.

13. Hofmann-Thiel, S., L. Turaev, and H. Hoffmann. 2010. Evaluation of the hyplex (R) TBC PCR test for detection of *Mycobacterium tuberculosis* complex in clinical samples. *BMC Microbiol.* **10:** 95.

14. Kraus, G., T. Cleary, N. Miller, R. Seivright, A. K. Young, G. Spruill, *et al.* 2001. Rapid and specific detection of the *Mycobacterium tuberculosis* complex using fluorogenic probes and real-time PCR. *Mol. Cell. Probes* **15:** 375−383.

15. Kubista, M., J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, *et al.* 2006. The real-time polymerase chain reaction. *Mol. Aspects Med.* **27:** 95−125.

16. Li, L., C. Mahan, M. Palaci, L. Horter, L. Loeffelholz, J. Johnson, *et al.* 2010. Sputum *Mycobacterium tuberculosis* mRNA as a marker of bacteriologic clearance in response to antituberculosis therapy. *J. Clin. Microbiol.* **48:** 46−51.

17. Mackay, I. M. 2004. Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* **10:** 190−212.

18. Mdivani, N., H. Li, M. Akhalaia, M. Gegia, L. Goginashvili, D. S. Kernodle, *et al.* 2009. Monitoring therapeutic efficacy by real-time detection of *Mycobacterium tuberculosis* mRNA in sputum. *Clin. Chem.* **55:** 1694−1700.

19. Philipp, S., H. P. Huemer, E. U. Irschick, and C. Gassner. 2010. Obstacles of multiplex real-time PCR for bacterial 16S rDNA: Primer specifity and DNA decontamination of Taq polymerase. *Transfus. Med. Hemother.* **37:** 21−28.

20. Piersimoni, C. and C. Scarparo. 2003. Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. *J. Clin. Microbiol.* **41:** 5355−5365.

21. Pounder, J. I., W. K. Aldous, and G. L. Woods. 2006. Comparison of real-time polymerase chain reaction using the Smart Cycler and the Gen-Probe amplified *Mycobacterium tuberculosis* direct test for detection of *M. tuberculosis* complex in clinical specimens. *Diagn. Micrbiol. Infect. Dis.* **54:** 217−222.

22. Rogers, G. B., F. A. Stressmann, G. Koller, T. Daniels, M. P. Carroll, and K. D. Bruce. 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. *Diagn. Microbiol. Infec. Dis.* **62:** 133−141.

23. Sontakke, S., M. B. Cadenas, R. G. Maggi, P. P. V. P. Diniz, and E. B. Breitschwerdt. 2009. Use of broad range 16S rDNA PCR in clinical microbiology. *J. Microbiol. Methods* **76:** 217−225.

24. Valasek, M. A. and J. J. Repa. 2005. The power of real-time PCR. *Adv. Physiol. Educ.* **29:** 151−159.

25. Van der Vliet, G. M., P. Schepers, R. Schukkink, B. Van Gemen, and P. R. Klatser. 1994. Assessment of mycobacterial viability by RNA amplification. *Antimicrob. Agents Chemother.* **38:** 1959−1965.

26. Young, G., S. Turner, J. K. Davies, G. Sundqvist, and D. Figdor. 2007. Bacterial DNA persists for extended periods after cell death. *J. Endod.* **33:** 1417−1420.