JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2008, p. 3177–3178 Vol. 46, No. 9
0095-1137/08/$08.00+0 doi:10.1128/JCM.00903-08

Molecular-Beacon-Based Real-Time PCR for Detection and Quantification of *Mycobacterium tuberculosis* DNA in Clinical Samples[▽]

Although real-time PCR (RT-PCR) has been extensively evaluated for diagnosis of *Mycobacterium tuberculosis* infections (5), data are limited on molecular-beacon (MB) applications. An MB-based RT-PCR protocol was designed and evaluated for direct *M. tuberculosis* detection and quantification in clinical specimens.

A total of 1,019 samples (417 pulmonary and 602 extrapulmonary) were consecutively and prospectively collected for tuberculosis (TB) diagnosis. They were processed using standard methodology (4) and divided into two parts. The first half of each sample was used for acid-fast staining and culture, while the second half was stored at −70°C. TB diagnosis of patients followed previous definitions (11). After diagnosis elucidation, the second part of each specimen obtained from TB-positive patients, as well as an equal number of specimens obtained from TB-negative patients, was thawed and DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

The RT-PCR targeted the IS*6110* sequence, and the following primers, amplifying a 161-bp fragment, and MB were designed, using Beacon Designer 5.1 software (Premier Biosoft, Palo Alto, CA): TB2F (5′-GTCCACGCCGCCAACTACG-3′), TB2R (5′-GTTAGGTGCTGGTGGTCCGAAG-3′), and TB2B (6-carboxyfluorescein–5′-CGCGATCGCCACAG CCCGTCCCGCCGATGATCGCG-3′-benzoic acid succinimidyl ester). Conditions consisted of 2 min of denaturation at 95°C, 50 cycles of 45 s of denaturation at 93°C, 90 s of annealing at 60°C, and 2 min of extension at 72°C, and finally, 7 min of extension at 72°C. DNA extracted from the *M. tuberculosis* H37Rv strain (ATCC 25618) was used for the quantification standard curve. DNA from an *M. avium* and an *M. chelonae* clinical isolate, from an *M. bovis* BCG strain, and from *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 49619, and *Bacteroides fragilis* ATCC 25285 strains was used for specificity confirmation. The median and mean of the quantified DNA and cycle threshold values were calculated using Instat 3.0 software (GraphPad Software, San Diego, CA 92130). A previously reported (8) single-step conventional PCR (C-PCR) was used for comparisons.

With the RT-PCR protocol, a positive signal was detected using DNA only from the *M. tuberculosis* and the *M. bovis* strains. RT-PCR analytical sensitivity was 7 fg of DNA, corresponding to approximately 1.4 mycobacterial genomes. Overall, 32 patients were TB positive, and 40 specimens were obtained from those patients (Tables 1 and 2). A total of 40 specimens (10 pulmonary and 30 extrapulmonary) were also obtained from 34 TB-negative patients. The two PCR assays showed equally high levels of sensitivity (Table 1), although C-PCR performed better among pulmonary specimens and RT-PCR performed better among extrapulmonary specimens. Combining the two assays yielded 100% sensitivity. After testing the samples diluted 10^{-1}, we found three samples to be inhibitory (one with both protocols and two with PCR only). DNA quantification results from comparisons of the main specimen types are shown in Table 2. The mean (± standard deviation) cycle threshold value for the RT-PCR was 33.5 (± 5.3; range, 19.3 to 40.7).

In the literature, TaqMan and linear fluorescence probes have mostly been applied for direct diagnosis of TB (1, 3, 5). MB technology has been evaluated for direct detection of *M. avium* (2, 6) as well as for detection of mutation-specific resistance in *M. tuberculosis* isolates (9, 10) and, in only one report, for direct TB diagnosis using pulmonary specimens (7). In the present study, a newly designed MB RT-PCR was developed and proved to be a promising tool, showing high sensitivity and specificity for a collection of pulmonary and extrapulmonary specimens, which in most cases (29 out of 40) were acid-fast staining and culture negative.

Our report has also shown that pulmonary and pus specimens had the highest median DNA loads, in contrast to the results seen with lymph nodes. Although lymph node biopsy is commonly used for extrapulmonary TB diagnosis, our results indicated that a specimen from the primal focal point of the disease, if retrievable, may yield higher mycobacterial loads.

TABLE 1. Sensitivities, specificities, and positive and negative predictive values of the two PCR assays

Clinical specimen category	PCR assay	% Sensitivity	% Specificity	% PPV[a]	% NPV[b]
Pulmonary (n = 9)	C-PCR	100	100	100	100
	RT-PCR	88.9	100	100	90.9
	C-PCR + RT-PCR	100	100	100	100
Extrapulmonary (n = 31)	C-PCR	90.3	100	100	90.9
	RT-PCR	93.5	100	100	93.8
	C-PCR + RT-PCR	100	100	100	100
Total (n = 40)	C-PCR	92.5	100	100	93.0
	RT-PCR	92.5	100	100	93.0
	C-PCR + RT-PCR	100	100	100	100

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.

TABLE 2. DNA quantification of the main specimen types by use of the RT-PCR protocol

Specimen category	RT-PCR quantification (fg)	
	Mean (± SD)	Median
Pulmonary[a] (n = 9)	20,287.51 (± 27,221.36)	5,900.00
Tissue biopsy (n = 8)	1,492.76 (± 3,052.54)	258.85
Pus (n = 7)	$25{,}982.00 \times 10^6$ (± $72{,}337.00 \times 10^6$)	3,747.00
Lymph node biopsy (n = 6)	23,641.12 (± 52,748.28)	4.90
Miscellaneous[b] (n = 10)	1,334.38 (± 2,567.68)	74.30

[a] Includes seven sputum and two lavage specimens.
[b] Includes three urine specimens, two bone marrow aspiration samples, two gastric fluid samples, one cerebrospinal fluid sample, one pericardial aspiration sample, and one prostatic specimen.

This study was funded in part by a grant from Pfizer Hellas.
There are no conflicts of interest to declare by any author.

REFERENCES

1. **Broccolo, F., P. Scarpellini, G. Locatelli, A. Zingale, A. M. Brambilla, P. Cichero, L. A. Sechi, A. Lazzarin, P. Lusso, and M. S. Malnati.** 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.
2. **Bruijnesteijn van Coppenraet, E. S., J. A. Lindeboom, J. M. Prins, M. F. Peeters, E. C. J. Claas, and E. J. Kuijper.** 2004. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. J. Clin. Microbiol. **42:**2644–2650.
3. **Cleary, T. J., G. Roudel, O. Casillas, and N. Miller.** 2003. Rapid and specific detection of *Mycobacterium tuberculosis* by using the Smart Cycler instrument and a specific fluorogenic probe. J. Clin. Microbiol. **41:**4783–4786.
4. **Delta-Latta, P.** 2004. Mycobacteriology and antimycobacterial susceptibility testing, p. 7.1.1–7.3.1. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, 2nd ed. ASM Press, Washington, DC.
5. **Espy, M. J., J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. C. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill III, and T. F. Smith.** 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. **19:**165–256.
6. **Fang, Y., W. H. Wu, J. L. Pepper, J. L. Larsen, S. A. E. Marras, E. A. Nelson, W. B. Epperson, and J. Christopher-Hennings.** 2002. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. J. Clin. Microbiol. **40:**287–291.
7. **Haldar, S., S. Chakravorty, M. Bhalla, S. De Majumdar, and J. S. Tyagi.** 2007. Simplified detection of *Mycobacterium tuberculosis* in sputum using smear microscopy and PCR with molecular beacons. J. Med. Microbiol. **56:**1356–1362.
8. **Kox, L. F. F., D. Rhienthong, A. Medo-Miranda, N. Udomsantisuk, K. Ellis, J. Van Leeuwen, S. Van Heusden, S. Kuijper, and A. H. J. Kolk.** 1994. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. J. Clin. Microbiol. **32:**672–678.
9. **Piatek, A. S., A. Telenti, M. R. Murray, H. El-Hajj, W. R. Jacobs, Jr., F. R. Kramer, and D. Alland.** 2000. Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob. Agents Chemother. **44:**103–110.
10. **Varma-Basil, M., H. El-Hajj, R. Colangeli, M. H. Hazbon, S. Kumar, M. Bose, M. Bobadilla-del-Valle, L. G. Garcia, A. Hernandez, F. R. Kramer, J. S. Osornio, A. Ponce-de-Leon, and D. Alland.** 2004. Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* isolates from India and Mexico by a molecular beacon assay. J. Clin. Microbiol. **42:**5512–5516.
11. **World Health Organization.** 2003. Treatment of tuberculosis: guidelines for national programmes, 3rd ed. WHO/CDS/TB/2003.313. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/cds_tb_2003_313/en/index.html. Accessed 7 May 2008.

Joseph Papaparaskevas*
Dimitra P. Houhoula
Argirios Siatelis
Athanassios Tsakris
Department of Microbiology
Medical School
National and Kapodistrian University of Athens
75 Mikras Asias Str.
11527 Athens, Greece

*Phone: 30-210-7462142
Fax: 30-210-7462143
E-mail: ipapapar@med.uoa.gr

▽ Published ahead of print on 16 July 2008.