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Development of a Real-Time qPCR Method for Detection and Enumeration of *Mycobacterium* spp. in Surface Water▽†

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A real-time quantitative PCR method was developed for the detection and enumeration of *Mycobacterium* spp. from environmental samples and was compared to two other methods already described. The results showed that our method, targeting 16S rRNA, was more specific than the two previously published real-time quantitative PCR methods targeting another 16S rRNA locus and the *hsp65* gene (100% versus 44% and 91%, respectively).

Water exposure (15) is one source of human infection caused by nontuberculous mycobacteria (NTM). Nevertheless, the isolation and enumeration of NTM from water is difficult because other microorganisms overgrow NTM colonies (22). Consequently, the development of an alternative detection and enumeration method is essential for monitoring NTM sources in the environment.

Two real-time quantitative PCR (qPCR) methods for NTM measurement have been described (7, 29). The primer pair used in the first real-time qPCR method (7) targets 16S rRNA and was previously used to track mycobacterial growth in industrial water samples by conventional PCR (31). It was presented as a sensitive test for members of the *Mycobacterium* genus because it detected 34 species of mycobacteria (19, 25). However, the primer specificity was only measured by conventional PCR against DNA of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* (31) or by *in silico* analysis (7). The second real-time qPCR method, targeting the *hsp65* gene (29), was also sensitive (detection of 34 out of 37 *Mycobacterium* spp. tested). Although the primers showed high specificity (no detection of 16 different nonmycobacterial species) by conventional PCR (21), their specificity combined with the qPCR probe was only tested against *Candida albicans* DNA (29).

We sought to develop a reliable real-time qPCR method to detect *Mycobacterium* spp. in water samples. The development involved *in silico* primer screening followed by a specificity study by conventional PCR. Furthermore, the efficiency (Ef), correlation coefficient (r^2), limit of quantification (LOQ), specificity (Sp), and sensitivity (Ss) of this new method targeting 16S rRNA were compared with those of the two previously described methods (7, 29).

DNA collection. Fifty nontarget microorganisms were isolated from surface water of the Seine River (Paris, France) and identified by sequencing of the bacterial 16S rRNA gene (MicroSeq 500 kit) or fungal 28S rRNA gene (D2 large-subunit rRNA kit) using an ABI Prism 3100 genetic analyzer (Applied Biosystems) (14, 24, 27). Sequences were analyzed with the Mega BLAST algorithm and submitted to GenBank under the accession numbers GU265670 to GU265719. Reference microorganisms phylogenetically distant from the *Mycobacterium* genus, such as *Helicobacter* sp., were included in the nontarget collection. Reference microorganisms closely related to the *Mycobacterium* genus, such as *Corynebacterium*, *Nocardia*, and *Rhodococcus*, which together with *Mycobacterium* belong to the CNM (corynebacteria, nocardia, and mycobacteria) group, were also included in the nontarget collection. The sensitivities of the real-time qPCR methods were estimated using 30 species of the *Mycobacterium* genus (see Table S1 in the supplemental material) isolated from clinical cases or surface water (22). After growth, colonies were suspended in 1× TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA). DNA was extracted as previously described (22), and the DNA concentration was estimated on the basis of absorbance at 260 nm and 280 nm using a WPA Biowave DNA spectrophotometer (Isogen Life Science).

Real-time qPCRs. Reactions were performed using an ABI 7500 real-time PCR system (Applied Biosystems). The Sybr green and TaqMan real-time qPCR assays were performed using qPCR MasterMix plus for Sybr green I low 6-carboxy-X-rhodamine (ROX) and qPCR MasterMix plus low ROX, respectively (Eurogentec). The TaqMan probes (Table 1) were labeled (Eurogentec) with the fluorescent dyes 6-carboxyfluorescein (5′ end) and Black Hole Quencher (3′ end). All reac-

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TABLE 1. Descriptions of the protocols used in this study in order to quantify *Mycobacterium* spp. by qPCR

Parameter[a]	B-HN[b]	Specification in qPCR method[c]:				
		A	B	C	D	E
Combination	NA[d]	With B-HN	Without B-HN	Without B-HN	NA	NA
Chemistry	PCR	TaqMan	TaqMan	TaqMan	Sybr green	TaqMan
F primer	I571R/110F (100 nM)	I571R/110F (100 nM)	110F (300 nM)	110F (900 nM)	pMyc14 (500 nM)	65kDaf2 (1 μM)
R primer	264R (10 nM)	I571R (1 μM)	I571R (300 nM)	I571R (300 nM)	pMyc7 (500 nM)	65kDar3 (1 μM)
Probe	NA	H19R (100 nM)	H19R (100 nM)	H19R (50 nM)	NA	Genus (0.3 μM)
No. of cycles	30	40	40	40	40	50
Denaturation	95°C, 45 s	95°C, 15 s	95°C, 15 s	95°C, 15 s	94°C, 30 s	95°C, 15 s
Annealing	65°C, 30 s	55°C, 20 s	55°C, 20 s	55°C, 20 s	63°C, 15 s	51°C, 15 s
Extension	72°C, 1 min	72°C, 40 s	72°C, 40 s	72°C, 40 s	72°C, 40 s	72°C, 40 s

[a] F, forward; R, reverse. Polymerase activation was performed at 95°C for 10 min before all amplification reactions.
[b] B-HN, balanced heminested PCR method described by García-Quintanilla et al. (11).
[c] Concentrations of forward primers, reverse primers, and probes are displayed in parentheses. qPCR methods A, B, and C are those developed in this study and based on the primers and probe designed by García-Quintanilla et al. (12); qPCR methods D and E are those proposed by Dutil et al. (7) and Tobler et al. (29), respectively.
[d] NA, not applicable.

tions were performed in a 25-μl reaction mixture volume in triplicate (2.5 μl of DNA). Determinations of cycle threshold (C_T) were performed by setting the instrument's threshold line at 0.1 ΔRn units (fluorescence gain above the baseline divided by the ROX channel signal).

To assess the performance of the real-time qPCR methods, we calculated the Ef, r^2, LOQ, Sp, and Ss for each method. Concerning Ef, r^2, and LOQ, 5-fold dilutions of *Mycobacterium chelonae* strain ATCC 35752^T DNA were prepared in three independent series, in order to achieve relative quantification by qPCR. The Ef was calculated as previously described (26), and the r^2 was calculated using SDS software (Applied Biosystems). Nonreproducible amplification was not taken into account to estimate the Ef and r^2. The LOQ was determined by the smallest DNA quantity detected for each assay. DNA quantities were calculated as the number of *M. chelonae* genome equivalents (GE) based upon the *M. chelonae* genome weight (4.4 fg) (9) possessing single copies of the 16S rRNA (32) and *hsp65* (17) genes. Sensitivity was defined as the percentage of *Mycobacterium* species which were detected, and specificity was defined as the percentage of nontarget microorganisms which were not detected according to the collection assessed.

Steps of development. The following 18 forward/reverse primer pairs were selected and tested *in silico* for sensitivity and specificity: SodF/SodR (6), Z261/Z212 (35), recF1/recR1 and recF3/recR2 (1), RPO5V/RPO3V (3), R5/RM3 (16), mycF/mycR (20), 8FPL/1492 (30), 110F/264R (12), 285F/264R (18), F246/R266-267 (2), WuF/WuR (34), 110F/I571R (12), MYC-12/MYC13 (5), GyrbA/GyrbE (4), F119/R184T7 (10), Pri9/Pri8 (4), and Tb11/Tb12 (28). Based on query coverage of the 100 first results, the theoretical specificities and sensitivities of the primers were checked using the GenBank Mega BLAST algorithm. This screening allowed the identification of 8 primer pairs whose *in vitro* specificity was tested using conventional PCR (23). Prior to the PCRs, the absence of PCR inhibitors in extracted DNA was checked using bacterial (8F/1512R) or fungal (ITS1/ITS4) universal primers (8, 33). We then developed a real-time qPCR method (TaqMan) using 5′-exonuclease fluorogenic PCR (12) and the most specific primer pair out of the 8 pairs tested using conventional PCR. We first compared our method with two previously published methods using the same primers, one including a balanced heminested (B-HN) PCR (method A) and one without (method B) (11) (Table 1). The primer titration matrix, primer-probe ratio matrix, and $MgCl_2$ adjustment matrix were determined based on the results of the comparison and following the manufacturer's recommendations (Eurogentec). The new optimized real-time qPCR method (method C) was compared with the two methods of qPCR (method D [7] and method E [29]) previously described (Table 1).

Primer pair selection. From among the 18 primer pairs initially evaluated, 8 primer pair candidates were selected based on their *in silico* sensitivities and specificities for mycobacterial DNA amplification (data not shown). Among the 8 selected primer pairs, primer pairs 110F/I571R and F119/R184T7 were the most specific toward *Mycobacterium* spp. using conventional PCR (Table 2). F119/R184T7 detected 2 genera of the CNM group (2 of 3 *Nocardia* spp. and 1 of 2 *Rhodococcus* spp.), and 110F/I571R detected only 1 genus of the CNM group (1 of 3 *Corynebacterium* spp.) but also detected 3 unrelated genera (1 of 1 *Flavobacterium* sp., 1 of 5 *Bacillus* spp., and 1 of 4 *Aeromonas* spp.). The amplification products (about 475 bp) from strains not related to *Mycobac-*

TABLE 2. *In vitro* specificity of 8 primer pairs selected from *in silico* studies and tested with conventional PCR amplification of nontarget microorganisms' DNA

Primer pair	No. of amplifications that were[a]:			% Specificity
	High	Low	Negative	
gyrBA/gyrBE	56	1	0	0.0
Z261/Z212	24	23	10	17.5
FSodF/RSodR	21	12	24	42.1
F246/R266267	8	24	25	43.9
MycF/MycR	12	13	32	56.1
Tb11/Tb12	9	3	45	78.9
110F/I571R	1	3	53	93.0
F119/R184T7	3	0	54	94.7

[a] High amplification corresponds to PCR product signals as bright as that of the positive control *M. chelonae* ATCC 35752^T, and low amplification corresponds to PCR product signals less bright than that of this positive control or with a different molecular size.

TABLE 3. Comparison of the efficiency, correlation coefficient, limit of quantification, sensitivity, and specificity of *Mycobacterium* isolate quantification by the three methods

Parameter[a]	Value obtained ($n = 3$) using qPCR method[b]:		
	C	D	E
% efficiency (mean ± SD)	74.3 ± 1.7	74.4 ± 3.3	74.2 ± 2.0
r^2 (mean ± SD)	98.6 ± 0.2	98.8 ± 0.0	98.3 ± 0.9
LOQ range (fg)	346–1731	69–346	69–346
LOQ range (GE)	79–393	16–79	16–79
Maximum C_T (mean ± SD)	38.4 ± 0.2	35.0 ± 1.5	38.2 ± 1.4
No. of *Mycobacterium* species detected (upper LOQ for 30 species tested)	23	30	30
Sensitivity (%)	77	100	100
No. of nontarget genera detected (upper LOQ for 24 genera tested)	0	13	2
Specificity (%)	100	44	91

[a] r^2, correlation coefficient; LOQ, limit of quantification; GE, DNA quantities expressed in number of *M. chelonae* genome equivalents.
[b] qPCR method C is the method developed in this study based on the primers and probe designed by García-Quintanilla et al. (12); qPCR methods D and E are the methods proposed by Dutil et al. (7) and Tobler et al. (29), respectively.

terium spp. were less intense than that of the positive control. The intensities of the amplification products from the CNM group were comparable to that of the positive control. Primer pair 110F/I571R seemed the best candidate to develop a specific real-time qPCR method based on TaqMan chemistry, since a probe (H19R) was previously designed to be used with primer pair 110F/I571R (12), whereas the design of a probe between primers F119 and R184T7 would have been difficult because the amplified region is too polymorphic among mycobacteria (10).

Influence of B-HN PCR. According to a previous study (13), the LOQ of method B (393 to 1,967 GE) was higher than that of method A (79 to 393 GE). However, our results also showed that B-HN PCR (method A) does not maintain constant values of Ef (58.7% ± 16.0%) or high values of r^2 (74.8 ± 0.0) in comparison to those obtained with the single step of method B (Ef = 68.5% ± 1.5% and r^2 = 96.7 ± 0.0). Consequently, the real-time qPCR method we developed was without B-HN PCR. It was optimized (method C) with regard to the primers, probe, and $MgCl_2$ concentration (Table 1). Method C reached the same LOQ (Table 3) as was estimated using qPCR with B-HN PCR (method A).

Comparison of real-time qPCR methods. The reproducible values for Ef and r^2 suggest that real-time qPCR methods C, D, and E detected *M. chelonae* equally well (Table 3). The LOQ values of methods D and E were lower than that of method C (Table 3). Method C detected 23 out of 30 *Mycobacterium* isolates tested even when 50 ng of target DNA was used (data not shown), whereas methods D and E detected all of the isolates (see Table S2 in the supplemental material). However, primers 65Darf2 and 65kDar3 used in method E did not detect isolates of *M. celatum*, *M. heckeshornense*, and *M. leprae* which were not taken into account in our study (29).

None of the 57 nontarget microorganisms were detected by method C, whereas methods D and E yielded PCR products for 13 and 2 different genera, respectively (see Table S3 in the supplemental material). Although Veillette et al. (31) did not detect *Pseudomonas* sp., *Escherichia* sp., or *Staphylococcus* sp. using conventional PCR, 2 of 4 *Staphylococcus* isolates yielded PCR products by qPCR when method D was used (see Table S3 in the supplemental material). The detection limits of conventional PCR, which are known to be lower than those of real-time qPCR, could explain the poor specificity that we have observed with method D compared to previous conventional PCR results (31). Using primers pMyc7 and pMyc14, Kox et al. (19) observed that *Corynebacterium*, *Nocardia*, and *Rhodococcus* isolates were detected by conventional PCR. The specificity of method D (7) might be improved by using TaqMan chemistry, primer pair pMyc7/pMyc14, and a probe such as the *Mycobacterium* genus probe (pMyc5a) designed by Kox et al. (19). *Rhodococcus* isolates were detected by method E, and 2 out of 4 representative strains of the *Bacillus* genus were detected within the LOQ (see Table S3 in the supplemental material).

To conclude, our new method C is more specific than methods D and E, whereas methods D and E are more sensitive than the method described here (Table 3). Our method appears to be the first real-time qPCR method that is totally specific for the *Mycobacterium* genus. Because low detection limits can be overcome by using a larger quantity of the sampling water, specificity is the critical control point for environmental methods.

Nucleotide sequence accession numbers. The sequences obtained in this study were submitted to GenBank under accession numbers GU265670 to GU265719.

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