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Internally Controlled Real-Time PCR Method for Quantitative Species-Specific Detection and *vapA* Genotyping of *Rhodococcus equi*†

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We developed a novel quantitative real-time PCR (Q-PCR) method for the soil actinomycete *Rhodococcus equi*, an important horse pathogen and emerging human pathogen. Species-specific quantification was achieved by targeting the chromosomal monocopy gene *choE*, universally conserved in *R. equi*. The *choE* Q-PCR included an internal amplification control (IAC) for identification of false negatives. A second Q-PCR targeted the virulence plasmid gene *vapA*, carried by most horse isolates but infrequently found in isolates from other sources. The *choE*-IAC and *vapA* assays were 100% sensitive and specific as determined using 178 *R. equi* isolates, 77 nontarget bacteria, and a panel of 60 *R. equi* isolates with known *vapA*$^+$ and *vapA*-negative (including *vapB*$^+$) plasmid genotypes. The *vapA*$^+$ frequency among isolate types was as follows: horse, 85%; human, 20%; bovine and pig, 0%; others, 27%. The *choE*-IAC Q-PCR could detect up to one genome equivalent using *R. equi* DNA or 100 bacteria/ml using DNA extracted from artificially contaminated horse bronchoalveolar lavage (BAL) fluid. Quantification was linear over a 6-log dynamic range down to ≈10 target molecules (or 1,000 CFU/ml BAL fluid) with PCR efficiency *E* of >0.94. The *vapA* assay had similar performance but appeared unsuitable for accurate (*vapA*$^+$) *R. equi* quantification due to variability in target gene or plasmid copy number (1 to 9). The dual-reaction Q-PCR system here reported offers a useful tool to both medical and veterinary diagnostic laboratories for the quantitative detection of *R. equi* and (optional) *vapA*$^+$ "horse-pathogenic" genotype determination.

Rhodococcus equi is a soil-dwelling actinomycete of the mycolata group that causes pyogranulomatous infections in the lungs and other different body locations in a variety of animal hosts. This facultative intracellular parasite is well known in veterinary medicine as the causal agent of foal pneumonia, a severe purulent bronchopneumonic infection with high case-fatality rates. The disease is recognized in many countries as the leading cause of mortality in foals and is a cause of serious concern to the equine industry as it can become endemic in stud farms and there is no effective vaccine for its prevention (9, 23). In recent years *R. equi* has emerged as an opportunistic human pathogen, especially in individuals infected with human immunodeficiency virus. In the human host the infection presents usually as tuberculosis-like cavitary pneumonia or bacteremia (2, 36). *R. equi* is also being increasingly reported in other animal species, mainly associated with extrapulmonary, purulent, caseating infections (5, 33). In cattle the organism is typically isolated from chronic retropharyngeal, bronchial, or mediastinal pyogranulomatous lymphadenitis (6) and in pigs from submaxillary lymph nodes (18).

Horse isolates of *R. equi* typically harbor an 85- to 90-kb virulence plasmid, of which an example has been fully sequenced (11, 32). This plasmid encodes virulence-associated protein A or VapA, a 17.4-kDa surface lipoprotein presumed to be involved in pathogenesis but whose role in the infectious process remains unknown (10, 14). VapA is encoded by the *vapA* gene, which is present in a plasmidic, 27.5-kb pathogenicity island together with six other *vapA* homologues (32). In nonhorse *R. equi* isolates, including human isolates, the VapA protein/*vapA* gene is much less frequently found than a variant protein/allele designated VapB/*vapB*. The VapB antigen is structurally and immunologically closely related to VapA but is larger (18.2 to 20 kDa as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting) and is encoded by plasmids of various sizes (79 to 100 kb), not yet characterized genetically. The *vapB* plasmids are not found in equine isolates, suggesting that *vapB*$^+$ strains are not pathogenic for the horse (22, 31, 34). Except for soil isolates from horse breeding farms, in which *vapA*-type plasmids are common, environmental isolates of *R. equi* do not usually carry plasmids, or if they do, these are smaller in size and most often *vapA* and *vapB* negative (31).

Laboratory diagnosis of rhodoccocal infections currently relies on classical bacteriological methods involving the isolation

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of the organism from clinical samples or postmortem material (23). However, these culture-based procedures are lengthy and sometimes lack adequate sensitivity due either to prior antibiotic treatments or, in the case of respiratory specimens (typically bronchoalveolar lavage [BAL] aspirate or sputum), to the presence of multiple bacterial contaminants (28). An added problem is the difficulties posed by the identification due to the micro- and macroscopic morphological variability exhibited by these bacteria and the lack of accuracy of biochemical tests for *R. equi* species determination (8, 15, 29, 36). There is therefore considerable interest in developing new, simpler tests for the rapid and reliable detection and identification of *R. equi* for use in both veterinary and medical clinical microbiology laboratories.

Several molecular methods for *R. equi* have been described based on amplification of DNA sequences by conventional PCR (1, 3, 12, 15, 22, 28, 30). Although comparatively faster than culture-based methods, conventional PCR, however, provides only qualitative results and requires post-PCR procedures. The "open" post-PCR processing of massive amounts of amplicon increases the risk of false-positive results due to sample cross-contamination. This risk is minimized in the real-time PCR technique as the reaction and fluorescent probe-based amplicon detection are brought about simultaneously in a closed tube. Real-time monitoring of the amplification curve via fluorescence emission permits also a much more sensitive detection of positive reactions and at the same time, importantly, an accurate quantification of the target DNA present in the sample (35). However, to date only one quantitative real-time PCR (Q-PCR) assay has been reported for *R. equi* (13). This assay targets the *vapA* gene and therefore detects only strains carrying $vapA^{+}$ plasmids, which as mentioned above are rarely found in human and most other nonhorse *R. equi* isolates, thus limiting its applicability to the field of equine medicine. Moreover, the quantification accuracy of this assay can be compromised by strain-to-strain differences in plasmid copy number or plasmid DNA extraction efficiency.

We recently identified the *R. equi* cholesterol oxidase gene *choE* and demonstrated that this chromosomal locus is universally conserved in these bacteria (21) and is a suitable target for their specific and sensitive detection by conventional PCR (15). Here we report the design and development of a novel dual-reaction Q-PCR method that allows both the species-specific quantification of *R. equi* and determination of its "horse-associated" subtype via detection of *choE* and *vapA* sequences, respectively. The method includes an internal amplification control (IAC) for monitoring the occurrence of false-negative results due to PCR failure or inhibition.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. A total of 255 bacterial strains were used in this study: 197 were *Rhodococcus* spp. (178 *R. equi* and 19 non-*R. equi* isolates) and 58 belonged to different actinomycete genera, including cholesterol oxidase-producing species. The *R. equi* strains included horse ($n = 81$), human ($n = 35$), pig ($n = 30$), bovine ($n = 8$), soil ($n = 13$), and ancillary ($n = 11$, from sheep, goat, dog, cat, pheasant, primate, iguana, and unknown origin) isolates from 14 countries (Argentina, Australia, Brazil, Canada, China, Dominican Republic, Germany, France, Hungary, Ireland, Japan, Slovenia, Spain, and the United Kingdom). All were confirmed as *R. equi* by analysis of colony morphology, API Coryne biochemical profiling, synergistic hemolysis (CAMP-like) test with *Listeria ivanovii* (21), and our previously described conventional *choE*-PCR test (15). *R. equi* isolate 103S from J. Prescott (University of Guelph, Canada), deposited as PAM 1126 in our collection, was used as the reference strain. This strain was originally isolated from a case of foal pneumonia and is currently being used for the determination of the complete genome sequence of *R. equi* by the International *R. equi* Genome Consortium (www.sanger.ac.uk/Projects/R_equi). A detailed list of *R. equi* isolates is available in the supplemental material as Table S1. The non-*R. equi* isolates are listed in Table S2 in the supplemental material. Bacteria were maintained at −80°C in a medium containing 2% tryptone, 4% skimmed milk, and 16% glycerol. *Rhodococcus* spp. were grown at 30°C in brain heart infusion (BHI) and non-*Rhodococcus* isolates at 37°C in YME medium (0.4% yeast extract, 1% malt extract, 0.4% glucose), supplemented with 1.5% agar for plate cultures. All media were purchased from Oxoid (Hampshire, United Kingdom), except BHI (from Difco-BD, Detroit, MI).

DNA isolation and quantification. Bacterial genomic DNA was isolated from overnight cultures on solid medium using a cetyltrimethylammonium bromide (CTAB)-based protocol. Bacterial colonies from half a petri dish were collected with a loop, suspended in 1 ml phosphate-buffered saline, pelleted at 4,000 × *g* for 10 min, and incubated for 1 h at 37°C after resuspension in 567 μl Tris-EDTA buffer and 3 μl 100-mg/ml (30,000 units) lysozyme (Sigma) solution. Subsequently, 30 μl 10% sodium dodecyl sulfate and 3 μl 20-mg/ml (1.8 units) proteinase K (Sigma) was added and the mixture was incubated again for 1 h at 37°C. Then, 170 μl 5 M NaCl, 80 μl CTAB-NaCl solution (10% CTAB in 0.7 M NaCl), and 5 μl 100-mg/ml (50 Kunitz units) RNase A (Sigma) were added followed by a 30-min incubation at 65°C. After cooling to room temperature, the mixture was extracted with phenol-chloroform and chloroform-isoamyl alcohol followed by DNA precipitation with isopropanol and washing with 70% ethanol (27). DNA was resuspended in 100 μl 10 mM Tris-HCl, pH 8.0, and its amount and quality were determined spectrophotometrically by calculating the ratio of optical density at 260 nm to that at 280 nm and visually by agarose gel electrophoresis.

Oligonucleotides. The oligonucleotide primers and TaqMan probes used in this study were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and purchased from Metabion AG (Martinsried, Germany). They are listed in Table 1. The IAC probe was labeled with HEX (6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein) and the *choE* probe with 6-carboxyfluorescein (FAM).

IAC construction. The IAC consisted of a 100-bp chimerical DNA containing a portion of the listeriolysin (*hly*) gene from *Listeria monocytogenes* (GenBank accession no. M24199), which we previously validated as a Q-PCR probe target (24), flanked by the *R. equi*-specific *choE* gene sequences targeted by reqF and reqR primers (Table 1). This chimeric DNA molecule was generated by two rounds of PCR as previously described (26). The first PCR used 1 ng *L. monocytogenes* DNA template and primers riacF and riacR (Table 1), which contained the corresponding *hly* target sequences plus a 5′ tail with the reqF or reqR primer sequence. The second PCR used the purified first-round PCR product (diluted 1:1,000) as a template and the reqF and reqR primers. PCR conditions were as previously described (26). The IAC PCR product was purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany), quantified, and diluted to the appropriate concentration in 10 mM Tris-HCl, pH 8.0, in the presence of 500 ng/ml of acetylated bovine serum albumin as a blocking agent to minimize binding of the negatively charged IAC DNA to the plastic microtubes. With the exception of its target sequence in the *L. monocytogenes hly* gene (nucleotide positions 114 to 177), the IAC did not display significant similarity to any DNA sequence deposited in public databases, as determined by BLAST-N searches (National Center for Biotechnology Information, Bethesda, Md.; http://www.ncbi.nlm.nih.gov). The IAC amplicon, 100 bp in size, was longer than the 68-bp *choE*-specific amplicon, facilitating the differentiation of the two PCR products by gel electrophoresis.

Q-PCR. The assays were performed essentially as described previously (24) in 20-μl reaction volumes containing 1× PCR buffer II; 6 mM $MgCl_2$; 200 μM dATP, dCTP, and dGTP; 400 μM dUTP; 300 nM specific primers; 150 nM probe (for the duplex *choE*-IAC system, 100 nM of IAC probe was added); 1 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, N.J.); 0.2 units of AmpErase uracil *N*-glycosylase; and 5 μl of the target DNA solution. Reactions were run on an iCycler IQ platform (Bio-Rad Laboratories Inc., Hercules, CA) with the following program: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Q-PCR results were analyzed using the Optical System Software v3.0a (Bio-Rad Laboratories Inc., Hercules, CA). Quantification was obtained by interpolation in a standard regression curve of cycle threshold (C_T) values generated from samples of known DNA concentrations. One molecule of *R. equi* DNA, or genome equivalent, corresponds to approximately 5.5 fg of DNA considering a genome

TABLE 1. Oligonucleotides used in this study

Target	Oligonucleotide name	Application	Sequence	T_m[a] (°C)	G-C (%)	Reference(s)
choE	reqF	Q-PCR forward primer	5′-CGA CAA GCG CTC GAT GTG-3′	59	61	This study
	reqR	Q-PCR reverse primer	5′-TGC CGA AGC CCA TGA AGT-3′	59	56	This study
	reqP	TaqMan probe	5′-FAM-TGG CCG ACA AGA CCG ATC AGC C-TAMRA[b]-3′	69	64	This study
	COX-F	PCR forward primer	5′-GTC AAC AAC ATC GAC CAG GCG-3′	62.3	57.1	15
	COX-R	PCR reverse primer	5′-CGA GCC GTC CAC GAC GTA CAG-3′	64.7	66.7	15
vapA	RvapA114F	Q-PCR forward primer	5′-CAG CAG TGC GAT TCT CAA TAG TG-3′	59	48	This study
	RvapA188R	Q-PCR reverse primer	5′-GAA GTC GTC GAG CTG TCA TAG CT-3′	59	52	This study
	RvapA140P	TaqMan probe	5′-FAM-CAG AAC CGA CAA TGC CAC TGC CTG-TAMRA-3′	69	58	This study
	IP1	PCR forward primer	5′-AC TCT TCA CAA GAC GGT-3′	46	50	22, 30
	IP2	PCR reverse primer	5′-TAG GCG TTG TGC CAG CTA-3′	55.1	55.6	22, 30
vapB	H1	PCR forward primer	5′-TGA TGA AGG CTC TTC ATA A-3′	47.6	36.8	22
	H2	PCR reverse primer	5′-TTA TGC AAC CTC CCA GTT G-3′	53.2	47.4	22
IAC	IACP	TaqMan probe	5′-HEX-CGC CTG CAA GTC CTA AGA CGC CA-TAMRA-3′	68	61	24
hly	riacF	Forward primer IAC construction	5′-CGA CAA GCG CTC GAT GTG CAT GGC ACC ACC-3′	81	63	This study
	riacR	Reverse primer IAC construction	5′-CGA CAA GCG CTC GAT GTG ATC CGC GTG TTT-3′	78	57	This study

[a] Theoretical melting temperature.
[b] TAMRA, 6-carboxytetramethylrhodamine.

size of 5.2 Mb as determined for PAM 1126, according to the following equation: DNA amount in fg = bp × 660 Da/bp × 1.6 × 10^{-27} kg/Da × 1 × 10^{-18} fg/kg (25). Q-PCRs with C_T values of >50 were considered negative. The 95% confidence interval was calculated for every serial dilution according to a binomial distribution (with the statistical software SPSS 12.0S for Windows v8.0 [SPSS Inc., Chicago, Ill.]) (25, 26). Unless otherwise stated, all reactions were performed in triplicate.

Quantitative detection of *R. equi* in BAL fluid. An overnight culture in BHI of *R. equi* PAM 1126 was centrifuged for 3 min at 3,000 × *g*, the bacterial pellet was resuspended in sterile phosphate-buffered saline, and the suspension was serially 10-fold diluted in BAL fluid obtained from a healthy adult horse with the use of 0.9% NaCl intravenous infusion solution (Baxter Healthcare Corp.) as vehicle. The concentration of *R. equi* in the BAL fluid dilutions was determined by standard plate counting. DNA was extracted from *R. equi*-contaminated BAL dilutions as follows: 1-ml samples were transferred to clean 1.5-ml microtubes and centrifuged for 5 min at 10,000 × *g* at 4°C; the pellets were resuspended in 100 μl Instagene Matrix suspension (Bio-Rad Laboratories, Hercules, CA) by vortexing, and the suspensions were incubated at 56°C for 20 min; after 10 s of vigorous vortexing, these were incubated at 100°C for 8 min and then placed on ice and centrifuged for 5 min at 14,000 × *g* at 4°C; finally, 50 μl of the supernatants was transferred to a fresh microtube and stored at −20°C until use. Each PCR used 5 μl of DNA preparation.

RESULTS

Design and optimization of *choE*- and *vapA*-specific Q-PCR assays. To specifically identify *R. equi*, we used the conserved *choE* gene (21) as a target. A previously developed conventional PCR assay based on detection of *choE* sequences was 100% specific and sensitive for *R. equi* taking as a positive result the expected 959-bp amplicon (15). Using this PCR assay, smaller products are occasionally observed with other rhodococcal species (reference 12 and our unpublished observations). To minimize the risk of unspecific reactions, we identified a new *choE* target region suitable for Q-PCR primers and probe design by careful analysis of all cholesterol oxidase gene sequences deposited in public databases using the CLUSTALW multiple-alignment tool (European Bioinformatics Institute, EMBL; www.ebi.ac.uk). The new *choE* primers, reqF and reqR (Table 1), amplify a 100% specific, conserved 68-bp DNA fragment corresponding to positions 938 to 1005 of the coding sequence deposited in GenBank under accession no. AJ242746 (21).

Gene regions suitable for *vapA*-specific Q-PCR oligonucleotides were selected by visual inspection of CLUSTALW multiple alignments of all known sequences of the *vap* multigene family. These include, in addition to *vapA*, six other *vap* genes (*vapC* to *-H*, identified in $vapA^+$ virulence plasmids from strains ATCC 33701 and 103) (32) and *vapB* identified in plasmids from "nonequine" *R. equi* isolates (22). Primer pair RvapA114F-RvapA188R amplifies a *vapA*-specific, conserved 75-bp DNA fragment corresponding to positions 114 to 188 of the gene sequence deposited in GenBank with accession no. NC002576 (32).

The BLAST-N tool v.2.2.12 was used (with default settings and low-complexity filter off) to confirm in silico that none of the selected oligonucleotides recognized any registered DNA sequence other than the target sequence. Primers, TaqMan probes, and $MgCl_2$ concentrations were optimized for Q-PCR assays by using 1 ng of template DNA from *R. equi* strain PAM 1126. The minimum primer and probe concentrations that gave the lowest C_T value and the highest fluorescence intensity were retained as the standard optimal conditions (see Materials and Methods).

Optimization of duplex *choE*-IAC Q-PCR assay. The optimal IAC probe concentration (i.e., the minimum concentration not resulting in an increase of C_T) (26), 100 nM, was experimentally determined by performing Q-PCRs in the presence of

1,000 IAC molecules, no *R. equi* DNA, 150 nM FAM-labeled *choE* probe, and increasing amounts (from 25 to 250 nM) of HEX-labeled IAC probe. Since an excess of IAC may inhibit the target-specific reaction, Q-PCRs were also carried out in the presence of various IAC amounts (10,000, 1,000, 100, and 10 molecules per reaction) and a fixed amount (30 genome equivalents) of *R. equi* PAM 1126 DNA. The maximum IAC amount with no inhibitory effect on the *choE*-specific FAM signal was 100 copies.

Specificity and sensitivity of the *choE*-IAC Q-PCR. The capacity of the *choE* Q-PCR assay to discriminate between target and nontarget bacteria was assessed using 1 ng of genomic DNA from 178 *R. equi* strains from a variety of sources (including clinical isolates from different animal species and environmental isolates), 19 non-*R. equi* rhodococcal species strains, and 58 strains from 18 different non-*Rhodococcus* actinomycete genera. The *choE* Q-PCR assay was 100% sensitive and 100% specific as all 178 *R. equi* strains tested gave a positive *choE* signal whereas none of the 77 nontarget bacteria did (detailed results in Table S1 in the supplemental material). *Rhodococcus fascians*, reported by others (12) to give an unspecific (smaller) amplicon by conventional PCR using our previously described primers (15), did not give any significant signal in the *choE* Q-PCR assay. All the reactions generated a positive IAC (HEX) signal, ruling out that the absence of *choE* (FAM) signal observed in non-*R. equi* isolates was due to failure of the PCR. All the nonactinomycete bacteria that we have tested to date, including a variety of common gram negatives and gram positives, have yielded negative results in the *choE* Q-PCR assay (not shown).

Specificity and sensitivity of the *vapA* Q-PCR. As with the *choE* PCR, none of the 77 nontarget bacteria (non-*Rhodococcus* strains and non-*R. equi* rhodococci) gave a positive amplification signal with the *vapA* Q-PCR. However, this assay detected the target sequence in only 48% of *R. equi* isolates (85 out of 178), as expected from the varied composition of the strain panel tested, which contained only a proportion of horse-derived bacteria (see above and Table S1 in the supplemental material). The distribution of *vapA*$^+$ isolates per animal species is shown in Fig. 1.

The discrimination capacity of the *vapA* Q-PCR assay was assessed on a selection of 60 *R. equi* strains using as reference method a previously described dual-reaction conventional PCR system that differentiates *vapA*$^+$ isolates from *vapB*-negative or *vapA*- and *vapB*-negative isolates using two pairs of primers (22). Prior to applying this method, we confirmed its 100% efficacy on a representative sample of *R. equi* strains with known *vapA*/*B* genotypes (17, 18). As shown in Table 2, there was a perfect concordance between the results obtained by the two techniques. As all the strains tested positive with the *choE*-IAC system, these results indicated that our *vapA* Q-PCR assay is 100% specific and 100% sensitive.

Detection and quantification limits of the *choE*-IAC and *vapA* Q-PCR assays. The detection and quantification limits of the developed PCR assays were determined by using *R. equi* PAM 1126 genomic DNA. Amplification reactions were performed with a range of DNA concentrations equivalent to approximately 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 10, and 1 target molecule. Figure S1 in the supplemental material illustrates typical amplification profiles and the regression

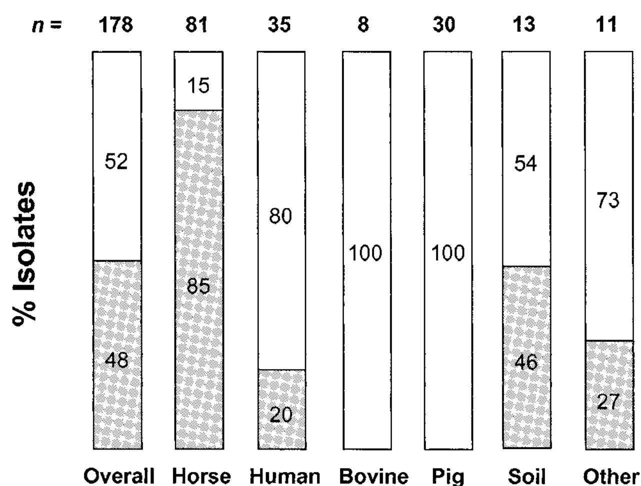

FIG. 1. Distribution of the *vapA* allele according to isolate origin. The number of isolates within each category is indicated above the bars; within the bars are the percentages of *vapA*$^+$ (gray section) and *vapA*-negative (empty section) isolates. "Other" includes sheep, goat, dog, cat, pheasant, primate, iguana, and unknown origin. Most soil isolates are from equine-related environments, explaining the relatively high percentage of *vapA*$^+$ isolates (31).

curves obtained with each Q-PCR assay; Table 3 shows the mean C_T values for a total of nine replicates in three independent experiments. The two Q-PCR assays yielded similar results in terms of absolute detection values. Positive amplification in all nine replicates of each DNA dilution was achieved when 10 or more target molecules were present, and as few as one target molecule could be detected with 67 to 78% probability for *choE*- and *vapA*-based Q-PCR assays, respectively (Table 3). The slopes of the linear regression curves calculated over a 6-log range were similar to the theoretical optimum of −3.32 (26) (*choE*, −3.337; *choE*-IAC duplex reaction, −3.335; *vapA*, −3.379) and showed that the amplifications were very efficient ($E = 0.994 \pm 0.001$, 0.995 ± 0.002, and 0.977 ± 0.002 for *choE*-, *choE*-IAC-, and *vapA*-based Q-PCR assays, respectively). Moreover, R^2 values were above the optimal 0.995 (0.998 for *choE* and *choE*-IAC reactions, 0.999 for *vapA* reaction), indicating that the Q-PCRs that we developed are appropriately linear. The confidence intervals based on the standard deviations of C_T values did not overlap each other down to 10 target molecules, indicating that reliable quantification was possible above this limit.

Quantitative detection of *R. equi* in BAL fluid. We assessed the applicability of the *choE*-IAC Q-PCR for the quantitative detection of *R. equi* bacteria in artificially contaminated BAL fluid. Taking into consideration that 100 μl of DNA was obtained from the processing of 1 ml of BAL fluid, and that 5 μl of DNA preparation was used for the PCR, our *choE*-IAC assay consistently detected down to approximately 100 *R. equi* cells/ml or 5 genomic units per reaction (Table 4). Quantitative amplification parameters were optimal, with linearity (R^2) above 0.99 down to 1×10^3 *R. equi* CFU/ml and overall PCR efficiency of 0.94. Relative accuracy values (24, 27) ranged between 88.95% and 113.53%, indicating a high degree of correspondence between the quantitative results obtained by the reference method (number of *R. equi* CFU/ml as

TABLE 2. Specificity and sensitivity of *vapA* Q-PCR[a]

Strain	Origin	Conventional PCR result[b]		Q-PCR result for *vapA*	Concordance[c]
		vapA	*vapB*		
PAM 1126	Horse	+	−	+	+
PAM 1286	Iguana	−	−	−	+
PAM 1335	Horse	+	−	+	+
PAM 1340	Horse	+	−	+	+
PAM 1346	Horse	+	−	+	+
PAM 1348	Soil	−	−	−	+
PAM 1350	Soil	+	−	+	+
PAM 1351	Soil	+	−	+	+
PAM 1358	Horse	+	−	+	+
PAM 1365	Horse	+	−	+	+
PAM 1367	Horse	+	−	+	+
PAM 1371	Horse	+	−	+	+
PAM 1374	Horse	+	−	+	+
PAM 1376	Human	−	+	−	+
PAM 1387	Unknown	−	−	−	+
PAM 1404	Horse	+	−	+	+
PAM 1406	Human	−	+	−	+
PAM 1408	Horse	+	−	+	+
PAM 1410	Horse	+	−	+	+
PAM 1413	Human	−	+	−	+
PAM 1414	Human	−	+	−	+
PAM 1415	Human	−	−	−	+
PAM 1416	Horse	+	−	+	+
PAM 1418	Horse	+	−	+	+
PAM 1422	Horse	+	−	+	+
PAM 1424	Horse	+	−	+	+
PAM 1425	Horse	+	−	+	+
PAM 1427	Horse	+	−	+	+
PAM 1430	Horse	+	−	+	+
PAM 1431	Horse	+	−	+	+
PAM 1436	Soil	+	−	+	+
PAM 1437	Horse	+	−	+	+
PAM 1441	Soil	−	−	−	+
PAM 1447	Pig	−	+	−	+
PAM 1448	Human	−	+	−	+
PAM 1453	Horse	+	−	+	+
PAM 1463	Human	−	−	−	+
PAM 1467	Pig	−	+	−	+
PAM 1468	Pig	−	−	−	+
PAM 1469	Pig	−	+	−	+
PAM 1473	Pig	−	+	−	+
PAM 1474	Pig	−	+	−	+
PAM 1475	Pig	−	+	−	+
PAM 1479	Pig	−	+	−	+
PAM 1480	Pig	−	+	−	+
PAM 1483	Pig	−	−	−	+
PAM 1485	Pig	−	−	−	+
PAM 1487	Pig	−	−	−	+
PAM 1488	Pig	−	−	−	+
PAM 1493	Pig	−	+	−	+
PAM 1495	Pig	−	+	−	+
PAM 1499	Pig	−	−	−	+
PAM 1500	Pig	−	+	−	+
PAM 1504	Pig	−	−	−	+
PAM 1518	Pig	−	−	−	+
PAM 1533	Pig	−	−	−	+
PAM 1547	Pig	−	−	−	+
PAM 1549	Pig	−	−	−	+
PAM 1550	Pig	−	−	−	+
PAM 1563	Bovine	−	−	−	+

[a] Note that $vapA^+$ and $vapB^+$ scores are mutually exclusive, indicating that *vapB* is most likely an allelic variant of *vapA*. +, positive; −, negative.

[b] Reference method (primers, PCR setup, and conditions as described in references 22 and 30).

[c] Concordance between results of *vapA* conventional PCR and our *vapA* Q-PCR.

determined by plate counting) and the results obtained by the *choE* Q-PCR method (Table 4).

DISCUSSION

We describe here a Q-PCR method that permits the sensitive and specific, accurate quantitative detection of the pathogenic actinomycete *R. equi*. This is achieved by targeting sequences from the chromosomal *choE* gene, previously identified in our laboratory and shown to provide a useful marker for the molecular detection and identification of *R. equi* (15, 21). A previous Q-PCR assay for *R. equi*, recently reported by others (14), targets the plasmidic gene *vapA* and thus detects only *R. equi* bacteria carrying this allelic variant. $vapA^+$ strains are associated with infections in the horse and hence are predominantly found in equine-associated specimens. However, *R. equi* can be isolated from a variety of other animal species in which, with few exceptions, $vapA^+$ strains are rarely found (17, 19, 22, 34) (Fig. 1). Indeed, our data show that only a small proportion (20%) of human clinical isolates are $vapA^+$, consistent with previously reported figures on the prevalence of $VapA^+/vapA^+$ *R. equi* bacteria in human specimens (12, 22, 34). The $vapA^+$ plasmid genotype appears to be particularly rare among bovine and pig isolates, as shown by our data (0% positives; Fig. 1) and other studies (6, 17). Overall, more than 50% of the isolates tested in this study were *vapA* negative (Fig. 1), clearly showing that a *vapA*-only-based detection assay misses a very significant proportion of common *R. equi* strains. Importantly, 15% of the horse clinical isolates included in our study were *vapA* negative (Fig. 1), questioning the value of *vapA* as a sensitive molecular diagnostic marker for *R. equi* even if its application is restricted to equine specimens.

Besides being universally highly conserved in *R. equi*, the *choE* gene offers also the advantage that it is present on the chromosome in monocopy (21), thus permitting an accurate quantification of the genomic units present in a sample. In contrast, the detection (and quantification in terms of genome equivalents) of a plasmidic gene, as is *vapA*, relies on the efficiency of plasmid DNA extraction and, critically, also on the number of copies of the plasmid carried by each individual strain. From the C_T values obtained for *choE* (control monocopy gene) and *vapA* we have estimated that 39% of the isolates contained two or more plasmid copies per genome (Table 5), indicating that Q-PCR data based on *vapA* would overestimate on a significant number of occasions the *R. equi* bacterial load present in the sample by at least a factor of two.

Although *vapA* is clearly unsuitable as a target for the species-specific quantitative detection of *R. equi* by Q-PCR, it is indisputable that this gene has diagnostic value as a predictor of horse pathogenicity (31). Moreover, horse isolates are quantitatively the most significant component of *R. equi* epidemiology. Indeed, this fully justifies the incorporation of *vapA* detection capabilities in any diagnostic method targeting *R. equi*. Bearing this in mind, we designed our Q-PCR method for *R. equi* as a dual-reaction system with independent assays for *choE* and *vapA*, the former as an entry-level, primary test aiming at the quantitative detection of *R. equi* species, and the latter as a complementary, optional test for $vapA^+$ genotype determination. This modular design provides full flexibility as

TABLE 3. Determination of the detection and quantification limits of *R. equi choE*, *choE*-IAC, and *vapA* Q-PCR assays[a]

Approx no. of genome eq/reaction	Confidence interval limit[b]		Signal ratio for assay[c]:			C_T for assay[d]:		
	Lower	Upper	*choE*	*choE*-IAC	*vapA*	*choE*	*choE*-IAC	*vapA*
1×10^6	997,600	1,003,300	9/9	9/9	9/9	19.83 ± 0.02	19.90 ± 0.04	18.80 ± 0.02
1×10^5	99,643	100,358	9/9	9/9	9/9	23.32 ± 0.05	23.40 ± 0.06	22.25 ± 0.06
1×10^4	9,887	10,113	9/9	9/9	9/9	26.05 ± 0.02	26.01 ± 0.03	25.60 ± 0.03
1×10^3	964	1,036	9/9	9/9	9/9	29.50 ± 0.02	29.60 ± 0.04	29.00 ± 0.03
1×10^2	89	111	9/9	9/9	9/9	32.88 ± 0.03	32.95 ± 0.05	32.20 ± 0.04
1×10^1	7	14	9/9	9/9	9/9	36.77 ± 0.15	36.80 ± 0.14	35.80 ± 0.12
1	0	2	6/9	6/9	7/9	38.38 ± 0.41	38.70 ± 0.34	38.20 ± 0.29

[a] Nontemplate controls were negative in the three Q-PCR assays (C_T values of >50 in all the replicates).
[b] Calculated for the expected number of template molecules at each dilution at 95% confidence level.
[c] Number of positive results out of nine reactions.
[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean values ± standard errors of the means were calculated with a prefixed threshold of 200. Differences in C_T values were statistically significant with $P < 0.05$.

the *vapA* Q-PCR assay will not always be needed, in particular in medical (human) microbiology laboratories, due to its specific relevance for the horse. It also helps avoid possible problems of loss of analytical performance, as is sometimes seen in multiplex PCR assays (7, 16). This is important as the primary *choE* Q-PCR test includes an IAC, thus being already de facto a duplex-format assay.

A major limitation to the application of Q-PCR-based tests in diagnostic laboratories is the relatively common occurrence of false-negative results due to the presence of PCR inhibitors in the sample. To tackle this problem, we included an IAC in our *choE* Q-PCR assay. An IAC consists of a nontarget DNA fragment that is coamplified with the target sequence, preferably with the same primers used for the test reaction (4, 26). To achieve this, we constructed the IAC by fusing the forward and reverse *choE* target sequences to both ends of an unrelated DNA fragment to which a second fluorescent probe (the IAC probe) hybridized. The use of two differently labeled fluorescent probes in the same reaction permitted the simultaneous detection/quantification of the target DNA and assessment of PCR efficiency. The inclusion of an IAC did not have any significant impact on the performance of the *choE* Q-PCR assay (Table 3).

A critical aspect in the design of molecular diagnostic methods for microbial pathogens is achieving low detection and quantification limits. This goal is of particular interest in the case of *R. equi*. Indeed, rhodococcal foal pneumonia initially follows an insidious course (9, 23), and it has been suggested that accurate detection (and quantification) of low levels of *R. equi* in respiratory specimens during the "silent" phase of infection, before the development of gross lesions in the lungs and the manifestation of clinical symptoms, could be of diagnostic value (13, 20). On purified DNA, our *choE* Q-PCR assay could detect approximately one target genome equivalent in at least 67% of the replicates and 10 genome equivalents in all cases. Accurate quantification, with excellent linearity (R^2 = 0.998) and efficiency (E = 0.994 without IAC and 0.995 with IAC), was possible down to ≈10 *R. equi* genome equivalents

TABLE 4. Quantitative detection of *R. equi* bacteria in BAL fluid[a]

Approx *R. equi* CFU/ml	Approx no. of *R. equi* genome eq/reaction[b]	Signal ratio[c]	C_T[d]	Relative accuracy[e]
1×10^7	5×10^5	9/9	20.67 ± 0.10	100.05
1×10^6	5×10^4	9/9	24.10 ± 0.10	103.59
1×10^5	5×10^3	9/9	27.82 ± 0.04	88.95
1×10^4	5×10^2	9/9	30.93 ± 0.13	113.53
1×10^3	5×10^1	9/9	34.68 ± 0.20	95.57
1×10^2	5×10^0	9/9	37.90 ± 0.21	NA[f]
1×10^1	5×10^{-1}	0/9	>50	NA
1	5×10^{-2}	0/9	>50	NA
0[g]	0	0/9	>50	NA

[a] Results from three independent experiments with three replicates each. Overall efficiency E is 0.94, and linearity R^2 is 0.99.
[b] Estimated number of *R. equi* genome equivalents in each PCR run assuming 100% DNA extraction efficiency (each reaction mixture contained 5 μl of a DNA preparation of 100 μl extracted from 1 ml BAL fluid).
[c] Number of positive results out of nine reactions.
[d] As defined in Table 3, footnote *d*.
[e] Degree of correspondence between the quantitative results obtained by standard plate counting (*R. equi* CFU/ml) and the results obtained by the *choE* Q-PCR method (27, 29).
[f] NA, not applicable.
[g] Noncontaminated BAL fluid.

TABLE 5. Numbers of copies of the *vapA* gene in *R. equi* isolates[a]

Avg no. of copies of *vapA* for value type		% of isolates
Exptl continuous range	Theoretical discrete	
0.57[b]–1.49	1	61.2
1.50–2.49	2	25.9
2.50–3.49	3	5.9
3.50–4.49	4	0
4.50–5.49	5	1.2
5.50–6.49	6	1.2
6.50–7.49	7	2.4
7.50–8.49	8	0
8.50–9.49	9	1.2
Total		100

[a] Estimated from Q-PCR results for *choE* and *vapA* in *vapA*$^+$ isolates (at least three replicates per reaction per isolate) using 1 ng of DNA and the following formula: number of *vapA* copies = $2^{-\Delta C_T}$, where $\Delta C_T = C_T^{vapA} - C_T^{choE}$, assuming 100% plasmid DNA extraction efficiency relative to chromosomal DNA. This calculation is possible because the PCR efficiencies for both targets, 0.977 and 0.995, were close to the optimal value $E = 1$, meaning that duplication of each amplicon occurs in each cycle across a wide linear range. The coefficient of variance was 7% and 11% for *choE* and *vapA* Q-PCR systems, respectively.
[b] Minimum value obtained (see Table S1 in the supplemental material).

per reaction. A similar performance was achieved when the technique was applied to the enumeration of bacterial cells in BAL fluid, a specimen commonly used in the clinical diagnosis of *R. equi* pneumonia (9, 28). Here, due to the DNA extraction protocol used, which involved processing of 1-ml BAL fluid samples, and the inclusion per reaction of a fraction of DNA extract (5 out of 100 μl), the practical detection limit for *R. equi* bacteria was 100 CFU/ml (or 5 genome equivalents per reaction). It should be possible to lower this detection limit by refining the sample processing so that DNA is extracted from larger specimen amounts and recovered in smaller volumes. Moreover, accurate determination of horse-pathogenic (i.e., *vapA*$^+$) *R. equi* numbers in environmental samples or in fecal or nasal specimens could also provide a predictive tool to assess the risk of *R. equi* clinical infection in stud farms where the organism is endemic. The optimal performance parameters of our *vapA* Q-PCR ($R^2 = 0.999$, $E = 0.977$) indicate that this assay is suitable for this purpose.

In conclusion, the dual-reaction Q-PCR method that we have developed for the rapid (results in less than 2 h), species-specific quantitative monitoring and determination of the *vapA*$^+$ (equine) subtype of *R. equi* provides a diagnostic tool potentially very useful in both medical and veterinary diagnostic laboratories.

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