Rapid Identification of *Rhodococcus equi* by a PCR Assay Targeting the *choE* Gene

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The actinomycete *Rhodococcus equi* is an important pathogen of horses and an emerging opportunistic pathogen of humans. Identification of *R. equi* by classical bacteriological techniques is sometimes difficult, and misclassification of an isolate is not uncommon. We report here on a specific PCR assay for the rapid and reliable identification of *R. equi*. It is based on the amplification of a fragment of the *choE* gene encoding cholesterol oxidase. The *choE*-based PCR was assessed by using a panel of strains comprising 132 isolates from different sources and of different geographical origins, all initially identified biochemically as *R. equi*, and 30 isolates of representative non-*R. equi* actinomycete species, including cholesterol oxidase producers. The expected 959-bp amplicon was observed only with *R. equi* isolates, as confirmed by sequencing of a variable region of the 16S RNA gene from a random sample of 20 PCR-positive isolates. All *R. equi* isolates gave a positive *choE*-based PCR result, which correlated with a high degree of conservation of the *choE* gene. Three of the 132 strains originally identified as *R. equi* were negative for the *choE* gene, and subsequent analysis of their 16S RNA gene sequences confirmed that they belonged to other bacterial species (*Dietzia maris*, *Mycobacterium peregrinum*, and *Staphylococcus epidermidis*). All non-*R. equi* isolates were negative by the *choE*-based PCR. ATCC 21387, the only known isolate of *Brevibacterium sterolicum*, gave a 959-bp amplicon whose DNA sequence was virtually identical to that of *R. equi choE*. Comparison of the 16S RNA genes indicated that ATCC 21387 should be considered an *R. equi* isolate.

The nocardioform actinomycete *Rhodococcus equi* is a primary pathogen of horses. In foals, *R. equi* causes severe pyogranulomatous pneumonia, often accompanied by ulcerative enteritis and mesenteric lymphadenitis (29). In recent years, *R. equi* has emerged as an important opportunistic pathogen in humans, causing potentially life-threatening infections in severely immunocompromised people, in particular, human immunodeficiency virus-infected patients (43). In humans, *R. equi* causes a lung disease reminiscent of pulmonary tuberculosis. *R. equi* can also infect cattle, in which it has been associated with ulcerative lymphangitis (34), and it has also been recovered from inflamed tonsils and the cervical lymph nodes of pigs (21, 34). The natural habitat of *R. equi* is the soil, especially that enriched with fecal material from domestic and wild animals (37).

R. equi infections are diagnosed by culturing and subsequent phenotypic analysis of the isolates by means of classical morphological and biochemical tests (9). However, the colony characteristics, cellular morphology, and reaction to acid-fast staining differ between *R. equi* isolates (32). Although the API Coryne multisubstrate identification system (bio-Merieux), a commercial kit widely used in clinical microbiology laboratories, includes *R. equi* in its database, its reliability for the biochemical identification of rhodococcal isolates is limited (8, 35). These inconsistent test results for *R. equi* frequently result in misidentification, in which *R. equi* is mistaken as other rhodococcal species or even corynebacteria or other actinomycetes (10, 16, 36). Accurate identification of *Rhodococcus* isolates to the species level is possible on the basis of chemotaxonomic properties (11). However, these techniques are excessively laborious, time-consuming, and expensive for routine use in clinical microbiology laboratories.

We recently identified the *R. equi choE* gene, a chromosomal locus encoding cholesterol oxidase (22), an enzyme believed to be a major virulence factor of *R. equi* (13). Mutational analysis indicated that ChoE is the membrane-damaging factor responsible for the typically shovel-shaped synergistic hemolysis (CAMP-like) reaction elicited by *R. equi* in the presence of sphingomyelinase C-producing bacteria, such as *Listeria ivanovii*, *Bacillus cereus*, and *Staphylococcus aureus* (22). This CAMP-like reaction can be used as a phenotypic marker for the rapid presumptive identification of *R. equi* (30; unpublished data). We describe here a new PCR method for the rapid and specific identification of *R. equi* based on the detection of *choE* sequences. This assay accurately differentiated *R. equi* from other closely related actinomycetes and correctly reassigned strains initially incorrectly identified as *R. equi* to other species on the basis of morphological and biochemical characteristics. It also identified as *R. equi* a well-known cholesterol oxidase-producing strain, *Brevibacterium sterolicum* ATCC 21387 (7).

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TABLE 1. Non-*R. equi* strains used in the study

Species	Strain
Corynebacterium sp.	ATCC[a] 14665
Corynebacterium kutscheri	ATCC 15677
Corynebacterium variabilis	ATCC 33010
Corynebacterium xerosis	NCIMB[b] 9956
Corynebacterium pseudotuberculosis	ATCC 19410
Corynebacterium bovis	CA[c] 23
Corynebacterium aquaticum	CA 29
Corynebacterium urealyticum	ATCC 43042
Mycobacterium sp.	NCIMB 11678
Mycobacterium phlei	ATCC 11758
Mycobacterium smegmatis	ATCC 14468
Mycobacterium bovis BCG	ATCC 35744
Mycobacterium xenopi	CA 78931
Mycobacterium kansasii	CA 70243
Mycobacterium tuberculosis	CA 1908
Mycobacterium terrae	CA 52828
Mycobacterium chelonae	CA 71765
Mycobacterium lentiflavum	CA 33805
Brevibacterium sp.	CA 157
Brevibacterium sterolicum	ATCC 21387
Nocardia sp.	CA 158
Nocardia asteroides	ATCC 14759
Rhodococcus sp.	NCIMB 9457
Rhodococcus erythropolis	ATCC 11048
Gordonia sp.	CA 93049
Gordonia bronchialis	ATCC 25592
Tsukamurella pulmonis	CIP[d] 104791[T]
Dietzia maris	ATCC 35013[T]
Turicella otitidis	CA 62716
Streptomyces sp.	CA 163

[a] ATCC, American Type Culture Collection.
[b] NCIMB, National Collections of Industrial and Marine Bacteria.
[c] CA, Collection of Actinomycetes of the Department of Biochemistry and Molecular Biology of the University of Cantabria. All strains were originally isolated from animal samples in the Animal Health Regional Laboratory, or from human patients in the Marqués de Valdecilla Hospital, Cantabria, Spain. They were identified by phenotypic analysis and 16S rDNA sequencing.
[d] CIP, Collection of Institut Pasteur, Paris, France.

MATERIALS AND METHODS

Bacterial strains and microbiological procedures. A total of 132 isolates from different geographical areas (Spain, France, Germany, the Dominican Republic, and Australia), identified at the source as *R. equi*, were included in the study. The 132 strains comprised 34 strains from pneumonic foals, 49 strains from human AIDS patients, and 49 strains from nonclinical samples (46 strains from soil and 3 strains from foal feces). Three *R. equi* reference strains, strains 103 (5), ATCC 6939 (the type strain of *R. equi*, also known as the Magnusson strain [18]), and ATCC 33701 (28), as well as 30 isolates of representative actinomycetes, were included as controls (Table 1). The bacteria were routinely cultured at 37°C for 48 h by using brain heart infusion as the base medium. Viable counts were determined by plating 10-fold serial dilutions of the initial bacterial suspension. CAMP-like synergistic hemolysis tests were performed on sheep blood agar plates with Columbia base medium (bioMérieux) and *L. ivanovii* ATCC 19119 as the indicator strain, as described previously (22, 30).

General DNA procedures. Bacterial genomic DNA was routinely prepared by suspending one colony in 50 µl of distilled water; the same volume of Instagene matrix (Bio-Rad) was added, and the mixture was heated at 80°C for 10 min. The samples were centrifuged at 10,000 × *g* for 5 min, and the supernatants were used for PCR amplification. Bacterial dilutions used in PCR sensitivity tests were processed in the same way. Highly purified, concentrated genomic DNA for use

in sensitivity tests was obtained as described previously (22). DNA concentrations were determined both by agarose gel electrophoresis and spectrophotometrically. Both strands of the PCR products were sequenced with an Applied Biosystems 377 apparatus. The sequences were analyzed by using the BLAST (1) network service from the National Center for Biotechnology Information (Bethesda, Md.) and were aligned by use of the AlignX program (InforMax, Bethesda, Md.). For restriction fragment length polymorphism (RFLP) analysis, the *choE* amplicons were purified by use of the Gel Extraction Purification kit (Qiagen) and were subsequently digested with the following restriction enzymes: *Ava*I, *Bam*HI, *Bgl*I, *Hin*PI, *Pvu*I, *Xho*I, and *Xmn*I (New England Biolabs, Beverly, Mass.). The digestion products were resolved by electrophoresis on 1.2% agarose gels in Tris borate buffer and were visualized by ethidium bromide staining.

Primers and PCR conditions. The oligonucleotide primers were synthesized by TIB MOLBIOL (Berlin, Germany). The reaction mixture for PCR amplification consisted of 10 µl of DNA template, 4 U of *Taq* DNA polymerase (Bioline, London, United Kingdom), 1.5 mM MgCl$_2$, 10 µl of 10× PCR amplification buffer [160 mM (NH$_4$)$_2$SO$_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween 20], 40 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate (Bioline), and double-distilled water to a final volume of 100 µl. The DNA was first denatured at 95°C for 5 min and was then subjected to 30 amplification cycles under the following conditions: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. After the final cycle, the reactions were terminated by an extra run at 72°C for 10 min, and then the mixtures were kept at 4°C until they were analyzed. All strains tested in this study were amplified at least three times.

Nucleotide sequence accession numbers. The 16S rRNA gene (rDNA) sequences from strains 20 and 21 are registered in the GenBank database under accession numbers AF537361 and AF537362, respectively.

RESULTS

R. equi-**specific PCR based on *choE*.** Oligonucleotide primers were designed after alignment of the sequence of *choE* from *R. equi* strain 103, recently determined in our laboratory (22), with other known or putative cholesterol oxidase gene sequences available in databases (*Mycobacterium tuberculosis*, GenBank accession no. X99343; *Mycobacterium leprae*, GenBank accession no. NC002677; *Streptomyces coelicolor*, GenBank accession no. AL161755; *Streptomyces* sp. *choA*, GenBank accession no. M31939; *Streptomyces* sp. *choM*, GenBank accession no. U13981; *Pimelobacter simplex*, GenBank accession no. AF247810; and *Burkholderia cepacia*, GenBank accession no. AB051407) to identify areas of similarity and difference. On the basis of that analysis we defined the 21-mer *R. equi*-specific primers COX-F (5′-GTCAACAACATCGACCAGGCG), corresponding to positions 1221 to 1241 (forward primer), and COX-R (5′-CGAGCCGTCCACGACGTACAG), complementary to the sequence spanning positions 2160 to 2180 (reverse primer) (coordinates are according to the sequence of the *choE* region deposited in the EMBL data bank under accession no. AJ242746). These primers are predicted to give an amplicon of 959 bp, according to the sequence of *R. equi* strain 103.

Genomic DNA from *R. equi* reference strains 103, ATCC 6939, and ATCC 33701 and from 132 isolates from different sources and of different geographical origins presumptively identified as *R. equi* were screened by PCR with the COX primers described above. For the 3 reference strains and for 129 of the presumptive *R. equi* isolates, the PCR resulted in the production of the expected 0.95-kbp product (Fig. 1). A region of the 16S rDNA comprising species-specific sequences was amplified by PCR with primers DG74 and PL06 (12) from a random sample of 20 of the *choE*-based PCR (*choE* PCR)-positive isolates and sequenced. These 20 strains included both
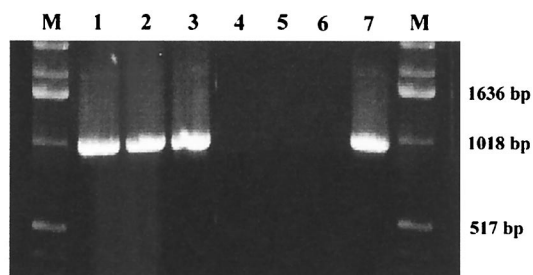
FIG. 1. Representative *choE* PCR amplification products obtained from genomic DNA samples of *R. equi*. Lanes: 1, strain 103; 2, ATCC 6939; 3, isolate 70; 4, isolate 11; 5, isolate 20; 6, isolate 21; 7, *B. sterolicum* ATCC 21387; M, DNA size marker.
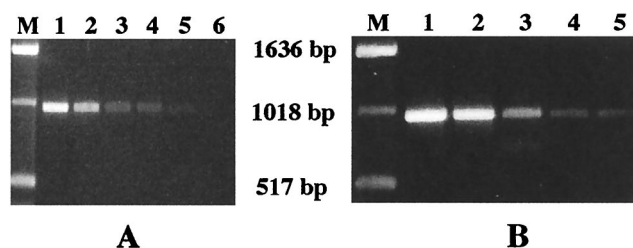

FIG. 2. Sensitivity of the *choE* PCR. (A) *choE* amplicon obtained with various amounts of purified *R. equi* ATCC 6939 genomic DNA. Lane M, DNA size marker; lanes 1 to 6, serial 10-fold dilutions of the DNA preparation (lane 1, 200 ng; lane 6, 2 pg). (B) *choE* amplicons obtained with various quantities of *R. equi* ATCC 6939 bacterial cells. Lane M, DNA size marker; lanes 1 to 5, 3×10^4, 3×10^3, 3×10^2, 30, and 3 CFU, respectively.

human and animal clinical isolates and environmental isolates. Their sequences were compared to the 16S rRNA sequences in databases, which confirmed that all the isolates belonged to *R. equi*. Only 3 of the 132 isolates (isolates 11, 20, and 21) gave a negative *choE* PCR result. The complete sequence of the 16S rDNA of each of these negative strains was determined after PCR amplification with primers 16S-F (5′-AGAGTTTGATC CTGGCTCAG) and 16S-R (5′-AGGAGGTGATCCAGCC GC). Strain 11 was identified as *S. epidermidis* (99% identity with *S. epidermidis* ATCC 14990[T] 16S rDNA), strain 20 was identified as *D. maris* (99% identity with *D. maris* ATCC 35013[T]), and strain 21 was identified as *M. peregrinum* (98% identity with *M. peregrinum* ATCC 14467).

The specificity of the *choE* PCR was verified by testing 30 strains from representative actinomycete species (Table 1). These strains included bacteria known to express cholesterol oxidase activity or to encode putative cholesterol oxidase enzymes, such as *Brevibacterium* spp., *Mycobacterium* spp., *Nocardia asteroides*, *Rhodococcus erythropolis*, and *Streptomyces* spp. (17). No amplification product was detected for any of these strains except one, *Brevibacterium sterolicum* ATCC 21387, for which a 0.95-kbp product, i.e., a product of the same size as that obtained with the *R. equi* isolates, was observed (Fig. 1). A further detailed analysis of the *B. sterolicum* strain indicated that it was missclassified and belonged to the *R. equi* taxon (see below).

In vitro sensitivity of *choE* PCR for *R. equi*. The in vitro sensitivity of the PCR test was assessed by determining both the minimum amount of DNA and the minimum number of bacterial cells required for detection of a 959-bp *choE* amplicon. For these tests, 10-fold dilutions of highly purified DNA or an exponential-phase culture (optical density at 600 nm = 0.5) of *R. equi* reference strains ATCC 6939 and ATCC 33701 were used. A positive result was obtained with a minimum of 0.02 ng of DNA or 3 *R. equi* CFU (Fig. 2).

Conservation of *choE* sequences in *R. equi*. The *choE* gene of strain 103 maps to a 2.3-kbp *Pst*I chromosomal fragment (22). Southern blot analyses revealed this same hybridization pattern in type strain ATCC 6939 and in the 20 *choE* PCR-positive isolates used for the 16S rDNA cataloguing described above (data not shown), suggesting a high degree of conservation of the *choE* sequence in *R. equi*. We confirmed this by DNA sequencing and RFLP analysis. The complete sequences of the *choE* genes from 3 of the 20 *R. equi* strains, selected on the basis of their different degrees of cholesterol oxidase and

cognate CAMP-like activities (strain 2, isolated from a diseased foal, had a high level of activity; strain 3, from a human patient, had an intermediate level of activity; and strain 70, also from a human patient, was exceptional, in that it presented no detectable activity), were determined. These three *choE* sequences diverged only minimally (sequence identities, >99.5%). RFLP analyses of the 959-bp *choE* amplicons from the 20 *R. equi* isolates previously analyzed by Southern blotting also revealed identical patterns for each of the seven restriction enzymes used (Fig. 3).

Reclassification of *B. sterolicum* ATCC 21387 as *R. equi*. Comparison of the cholesterol oxidase gene sequences of *R. equi* 103 (*choE*) and *B. sterolicum* ATCC 21387 (*choB*) (22) revealed they were almost identical, consistent with the positive result obtained with *B. sterolicum* ATCC 21387 by PCR with the COX primers (see above). This high degree of sequence conservation between *choE* and *choB* derived from two presumptively different bacterial species was surprising in view of the substantial degree of divergence (81%) that exists between cholesterol oxidase genes from different actinomycetes, even for those belonging to the same genus (as, for example, the case of *Streptomyces* sp. *choA* and *choM* alleles, which are only 86% identical). Given the strong sequence conservation of *choE* among *R. equi* isolates, the identity between *choE* and the reported *choB* sequence could be explained either (i) by a recent horizontal gene transfer event between *R. equi* and *B. sterolicum* or (ii) by the fact that ATCC 21387 is an *R. equi* isolate.

FIG. 3. RFLP patterns of *choE* amplicons from three *R. equi* isolates (strains 15, 65, and 115, of equine, human, and environmental origin, respectively, in that order for the digestions with each of the enzymes) digested with *Pvu*I (lanes 1 to 3), *Xho*I (lanes 4 to 6), *Xmn*I (lanes 7 to 9), *Ava*I (lanes 10 to 12), *Bam*HI (lanes 13 to 15), *Bgl*I (lanes 16 to 18), and *Hin*PI (lanes 19 to 21). Lane M, DNA size marker.

To discriminate between these two possibilities, we determined the complete sequence of the 16S rDNA of ATCC 21387 after PCR amplification with primers 16S-F and 16S-R (see above). The sequence was 100% identical to that of *R. equi* type strain ATCC 6939 (GenBank accession no. X80603), indicating that *B. sterolicum* ATCC 21387 is in fact an *R. equi* strain. ATCC 21387 gives a CAMP-like reaction with *L. ivanovii*. Furthermore, the biochemical profiles of ATCC 21387 determined with the API Coryne system are fully compatible with those of *R. equi* and less similar to those of *Brevibacterium.*

DISCUSSION

In this work, we studied the usefulness of the *R. equi* cholesterol oxidase gene, *choE*, recently identified and characterized in our laboratory (22), as a target for the development of a species-specific PCR method for the rapid identification of this pathogenic nocardioform actinomycete. Routine application of a PCR-based method requires that the target sequence be highly specific for the microorganism and that it be highly conserved in all strains of that organism. Our results show that the *choE* target sequence used fulfills these requirements. Sequences complementary to the COX primers were present in all of the isolates included in a large collection of *R. equi* strains from different sources and of different geographical origins, suggesting that they are universally conserved in this bacterial species. This is consistent with the production of extracellular *choE*-derived cholesterol oxidase activity and of its associated phenotypic marker, the CAMP-like reaction with *L. ivanovii*, by virtually all isolates of *R. equi* (unpublished data). On the other hand, no *choE* amplicon was detected in any of the 29 actinomycetes used as negative controls, which included relevant pathogenic species, such as *N. asteroides* and *M. tuberculosis*, that also produce cholesterol oxidase activity or that carry *choE*-related genes (17). Most importantly, these negative control strains included cholesterol oxidase-producing rhodococci, demonstrating the species specificity of our PCR method. The *choE* gene homologs carried by these bacteria were sufficiently divergent to prevent positive amplification with our COX primers. The specificities of these primers for *R. equi* were corroborated by sequence analysis of the 16S rDNA from a representative sample of the *choE* PCR-positive strains.

Interestingly, one *R. equi* isolate from the panel of isolates tested did not produce detectable CAMP-like activity but gave a positive *choE* PCR result. This illustrates the validity of the molecular method that we developed as a tool to identify *R. equi*, as it gives positive results even for rhodococcal strains that do not express the *choE*-associated phenotypic marker. The cholesterol oxidase-nonproducing *R. equi* isolate, strain 70, acquired the capacity to produce an active enzyme (and CAMP-like reactivity) upon complementation with a plasmid carrying a wild-type *choE* gene, indicating that that strain bears a nonfunctional *choE* allele. Analysis of the strain 70 *choE* sequence revealed the presence of a 1-bp insertion in the 5′ region of the gene that produced a frame shift and, subsequently, the loss of cholesterol oxidase activity (unpublished results).

The *choE* PCR with the COX primers also identified three isolates that had incorrectly been identified as *R. equi* according to the negative PCR results, thus further illustrating the

value of the assay. These three isolates were confirmed to be non-*R. equi* isolates by 16S rDNA sequencing. One, an isolate from the bone marrow of a human patient with human immunodeficiency virus infection, was identified as *D. maris*, a halophilic actinomycete previously reported as being involved in human infections only on two occasions (4, 27). The second isolate was identified as *M. peregrinum*, a species also rarely found as a cause of opportunistic infections in humans (25, 31). The third was a strain of *S. epidermidis*, a coagulase-negative *Staphylococcus* sp., which was possibly identified as *R. equi* on the basis of positivity by Gram staining, the coccoid aspect often presented by *R. equi*, and the resemblance of old *R. equi* colonies to those of staphylococci.

B. sterolicum ATCC 21387 was the only presumptive non-*R. equi* strain included in our study which yielded a 0.95-kb PCR product with the COX primers. ATCC 21387 is a well-known strain used for the industrial production of cholesterol oxidase and the extensive genetic and biochemical characterization of this enzyme (20, 23, 24). Indeed, the three-dimensional crystal structure of the cholesterol oxidase protein was determined by using the purified enzyme from *B. sterolicum* ATCC 21387 (15). Our previous data showed that the sequence of the cholesterol oxidase gene from *B. sterolicum* ATCC 21387, designated *choB*, was almost identical to that of *choE* from *R. equi* (22). Here we found that the 16S rDNA sequence of ATCC 21387 is also identical to that of *R. equi*. This finding, together with the strong conservation of the *choE* gene sequence among *R. equi* isolates, indicates that ATCC 21387, the only known strain of the species "*B. sterolicum*," was misclassified and is *R. equi*.

Only a few rapid molecular methods have been developed for *R. equi* identification. Clinical isolates from foals can be identified by detection of the VapA antigen, with monoclonal antibodies, or alternatively, by PCR detection of its gene, *vapA*, which is present on an 85-kb virulence plasmid (38, 39, 40). However, the virulence plasmid is not present in all strains of human and environmental origin (in general, in nonequine isolates) (41), thus limiting the general usefulness of the *vapA*-based identification of *R. equi* by PCR. Other PCR-based molecular methods have been used to amplify a chromosomal segment of unknown function (2) or the 16S rDNA (3, 33), but their validities have been assessed with only a small number of strains. Although 16S rDNA sequencing is accepted as a general means for species differentiation, some heterogeneity can exist between different isolates of the same species (14). A recent study of the 16S rDNA sequences of several representative strains showed that *R. equi* is a very heterogeneous taxon, with variations in 16S rDNAs of up to 4% (19). On the other hand, closely related species may have identical or almost identical 16S rDNA sequences (6, 26). Therefore, it is useful to have other species-specific targets, such as *choE*, to undertake an assay for the reliable identification of a bacterial species. Finally, a PCR-RFLP method targeting a 65-kDa heat shock protein gene and primarily devised for the identification of mycobacteria was shown to discriminate *R. equi* strains (42). However, this assay is too laborious and lengthy because, due to the conserved nature of the 65-kDa heat shock protein gene, the amplification product is of the same size for all actinomycete species and *R. equi* can be discriminated only by restriction analysis of the amplicon.

In summary, we describe a new PCR assay which can be usefully applied for the rapid, sensitive, and reliable identification of *R. equi* isolates and their differentiation from isolates of other pathogenic and nonpathogenic actinomycetes.

ACKNOWLEDGMENTS

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