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## Identification of Atypical *Rhodococcus*-Like Clinical Isolates as *Dietzia* spp. by 16S rRNA Gene Sequencing<sup>∇</sup>

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***Rhodococcus equi* and *Dietzia* spp. are closely related actinomycetes that show similar phenotypic properties. In humans, *R. equi* is an opportunistic pathogen associated with severe immunodeficiency. *Dietzia* spp. are environmental bacteria that have been isolated recently from clinical material and are presumptively associated with human infections. During the last 5 years, 15 bacterial isolates from human clinical samples collected at the Hospital Marqués de Valdecilla, Santander, Spain, were identified as *R. equi* by the API Coryne test. 16S rRNA gene sequencing confirmed seven isolates to be true *R. equi* strains, whereas the other eight were identified as members of the genus *Dietzia*, including *Dietzia maris* (four isolates), *Dietzia natronolimnaea* (two isolates), and *Dietzia timorensis* and *Dietzia* sp. (one isolate each). The eight *Dietzia* isolates were highly sensitive to 12 antimicrobial compounds.**

The nocardioform actinomycete *Rhodococcus equi* is a multihost pathogen that causes pyogranulomatous infections in a variety of animal species, with special significance in horses and humans (23). In immunocompromised persons, *R. equi* causes tuberculosis-like pneumonia associated with a high case-fatality rate, particularly in human immunodeficiency virus (HIV)-infected patients (22). The number of cases of infection associated with *R. equi* in humans increased with the expansion of the AIDS pandemic but in recent years has diminished due to the control of HIV spread.

*R. equi* infections are diagnosed by culture and subsequent phenotypic analysis of samples by means of classical morphological and biochemical tests (9). In clinical microbiology laboratories, *R. equi* is usually identified using the API Coryne system (bio-Mérieux, Marcy l'Étoile, France), a commercial multisubstrate kit that includes *R. equi* in its database. However, its reliability for the identification of rhodococcal isolates is limited, resulting in misidentification of *R. equi* as other rhodococcal species or even other actinomycetes (9). Several molecular methods based on amplification of DNA sequences by PCR for detection and identification of this pathogen have been proposed. Since *R. equi* is particularly relevant in equine medicine, most target the plasmid-borne *vapA* gene, encoding a virulence factor associated with horse pathogenesis. However, these methods are not useful for human isolates, because they generally do not carry *vapA*-type virulence plasmids but alternative types, like the pig-associated *vapB* plasmid and a

recently identified new bovine type, or are plasmid-less (16). We therefore developed a PCR method for species-specific *R. equi* identification based on the amplification of the *choE* gene (11, 20), a chromosomal locus encoding a secreted cholesterol oxidase (14). ChoE is the cytolytic factor responsible for the 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* (14). We use this CAMP-like reaction as a phenotypic marker for the rapid presumptive identification of *R. equi*. However, this functional test may miss *R. equi* isolates not expressing cholesterol oxidase despite having the *choE* gene or give false-positive results for other extracellular cholesterol oxidase-producing actinomycetes.

During the last 5 years, 15 bacterial isolates with phenotypic properties resembling those of rhodococci were collected from human specimens and isolated at the Clinical Microbiology Laboratory of the Hospital Marqués de Valdecilla, Santander, Spain. All of them were identified as *R. equi* by the API Coryne test. However, several of these isolates showed colony morphology and pigmentation characteristics that differed from those typical of *R. equi* strains. The 15 isolates were additionally tested with the CAMP assay and the *R. equi*-specific *choE* PCR assay, and their 16S rRNA genes were sequenced. Analysis of 16S rRNA gene sequences provides reliable identification at the species level for most of the clinically significant bacteria (6).

CAMP 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 (14). Highly purified genomic DNA samples from all the strains under study were prepared by following a previously described protocol for *R. equi* genomic DNA extraction (14). The *R.*

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TABLE 1. Identification of eight *Dietzia* isolates initially classified as *R. equi* or *Rhodococcus* sp.

Isolate <sup>a</sup>	Result of CAMP test (using <i>L. ivanovii</i> )	API Coryne identification	Result of <i>choE</i> -specific PCR assay	Length (bp) of 16S rRNA gene sequence	Identification based on 16S rRNA gene sequence (% identity to best match)	GenBank accession no. for 16S rRNA gene
CA138	+	<i>R. equi</i>	+	1,424	<i>D. maris</i> DSM 43672 (99.8)	GU247959
CA149	+	<i>Rhodococcus</i> sp.	–	1,467	<i>D. schimae</i> YIM 65001 (94.6)	GQ870422
CA150	+	<i>Rhodococcus</i> sp.	–	1,468	<i>D. maris</i> DSM 43672 (99.7)	GQ870423
CA155	–	<i>Gordonia</i> , <i>Dietzia</i> , or <i>Nocardia</i> species or <i>R. equi</i>	–	1,472	<i>D. timorensis</i> ID05-A0528 (99.6)	GQ870424
CA160	–	<i>Rhodococcus</i> sp.	–	1,465	<i>D. maris</i> DSM 43672 (99.9)	GQ870425
CA161	–	<i>Rhodococcus</i> sp.	–	1,433	<i>D. natronolimnaea</i> CV46 (99.5)	GQ870426
CA165	–	<i>R. equi</i>	–	1,451	<i>D. natronolimnaea</i> CV46 (100)	GQ870427
CA167	–	<i>R. equi</i>	–	1,373	<i>D. maris</i> DSM 43672 (100)	GQ870428
ATCC 616106	–		–		<i>D. maris</i>	
ATCC 6939	+		+		<i>R. equi</i>	

<sup>a</sup> ATCC 616106 and ATCC 6939 are reference type strains of *D. maris* and *R. equi*, respectively.

*equi*-specific *choE* PCR was carried out with COX primers as described in detail elsewhere (11). The universal primers PA (5'-AGAGTTTGATCTGGCTCAG-3') and PL06 (5'-GGTTAAGTCCCAGCAACGAGCGA-3') for the forward strand and PH (5'-AAGGAGGTGATCCAGCCGCA-3') and PLO6-R (5'-GC GCTCGTTGCGGGACTTAACC-3') for the reverse strand were used to generate overlapping DNA amplicons from the 16S rRNA genes. These amplicons were sequenced by primer walking using dye terminator chemistry in an Applied Biosystems model 377 apparatus. Homology searches were performed with the BLAST tool (1) at the website of the National Center for Biotechnology Information (Bethesda, MD; <http://www.ncbi.nlm.nih.gov>). The 16S rRNA gene sequences from the eight isolates identified as *Dietzia* species (Table 1) were registered in the GenBank database. A phylogenetic tree was inferred from the aligned sequences by the neighbor-joining method (21).

Ten of the 15 isolates gave a positive CAMP test result with *L. ivanovii*, whereas PCR amplification of the genomic DNA samples with COX primers gave positive results for only 8 isolates. Sequencing of the 16S rRNA genes from all isolates revealed that for eight isolates, the best match was a sequence from the genus *Dietzia*. The genus *Dietzia* has been established very recently and includes environmental isolates with great resemblance in morphology and colony appearance to *R. equi* (13). Several authors have reported the isolation of *Dietzia* strains, notably *Dietzia maris* (3, 18) but also *Dietzia papillomatosus* (12) and *Dietzia cinnamea* (25), from human clinical material. The 16S rRNA genes of isolates CA138, CA150, CA160, and CA167 showed >99% identity to the corresponding gene from *D. maris* strain DSM 43672 (Table 1), the type strain of the genus *Dietzia* (15). *D. maris* has been isolated from a variety of environments, including soils, deep sea mud, and the dinoflagellate *Pyrodinium bahamense* (2). It has also been recovered from the skin of healthy humans (8), and in four instances, it has been associated with human infections. Bemmer-Melchior et al. isolated *D. maris* from the blood of an immunocompromised patient suffering septic shock and carrying a long-term central line *in situ* (3). This *D. maris* isolate was susceptible to  $\beta$ -lactam agents, aminoglycosides, macrolides, pristinamycin, rifampin, trimethoprim-sulfamethoxazole, and vancomycin. *D. maris* was also identified as the causal agent of a prosthetic hip infection (18). This isolate was susceptible to

amoxicillin, imipenem, gentamicin, pristinamycin, trimethoprim-sulfamethoxazole, rifampin, clindamycin, and vancomycin. Reyes et al. isolated *D. maris* from the pericardial liquid and the aortic wall of a patient with aortitis (19). The isolate was susceptible to teicoplanin, tobramycin, linezolid, rifampin, imipenem, ciprofloxacin, gentamicin, vancomycin, clarithromycin, clindamycin, and amoxicillin-clavulanic acid. Finally, Broseta et al. isolated *D. maris* from the blood of a patient with cardiac and respiratory insufficiency (5).

The 16S rRNA genes of two isolates, CA161 and CA165, were >99% identical to that of *Dietzia natronolimnaea* CV46. *D. natronolimnaea* strains have been isolated from salt lakes, alkaline waters, and the guts of Japanese horned beetle (*Trypoxylus dichotomus*) larvae (13). Morphologically, colonies from isolate CA155 did not resemble those from the other five isolates. The CA155 16S rRNA gene was 99% identical to that from *Dietzia timorensis* ID05-A0528. This species was proposed to classify a soil isolate from West Timor, Indonesia, and has recently been validly recognized as a novel *Dietzia* species (24). This is the first reported isolation of the alkaliphilic *D. natronolimnaea* and the recently established species *D. timorensis* from human specimens. Finally, the 16S rRNA gene of isolate CA149 was most similar (94.6%) to that of *Dietzia schimae* YIM 65001. However, since the levels of identity of the CA149 16S rRNA gene to genes from *D. schimae* YIM 65001 and other *Dietzia* spp. registered in GenBank were lower than 99%, CA149 was assigned the identification *Dietzia* sp.

Figure 1 shows a phylogenetic tree based on 16S rRNA genes depicting the relationships among the eight *Dietzia* isolates identified in this study and representative strains of *Dietzia* and *R. equi*. Isolate CA149 forms a detached branch in the tree, whereas the other isolates group with a representative *Dietzia* strain.

Previous reports by other authors and our own data suggest that dietziae have the potential to act as opportunistic pathogens. To date, no comprehensive investigation of the susceptibilities of human *Dietzia* isolates to antibiotics has been carried out. The *in vitro* susceptibilities of the eight identified *Dietzia* bacteria to antimicrobial agents were evaluated by the Etest diffusion gradient method according to the instructions of the Etest manufacturer (AB Biodisk, Solna, Sweden). The guidelines of the CLSI for testing the antibiotic susceptibilities

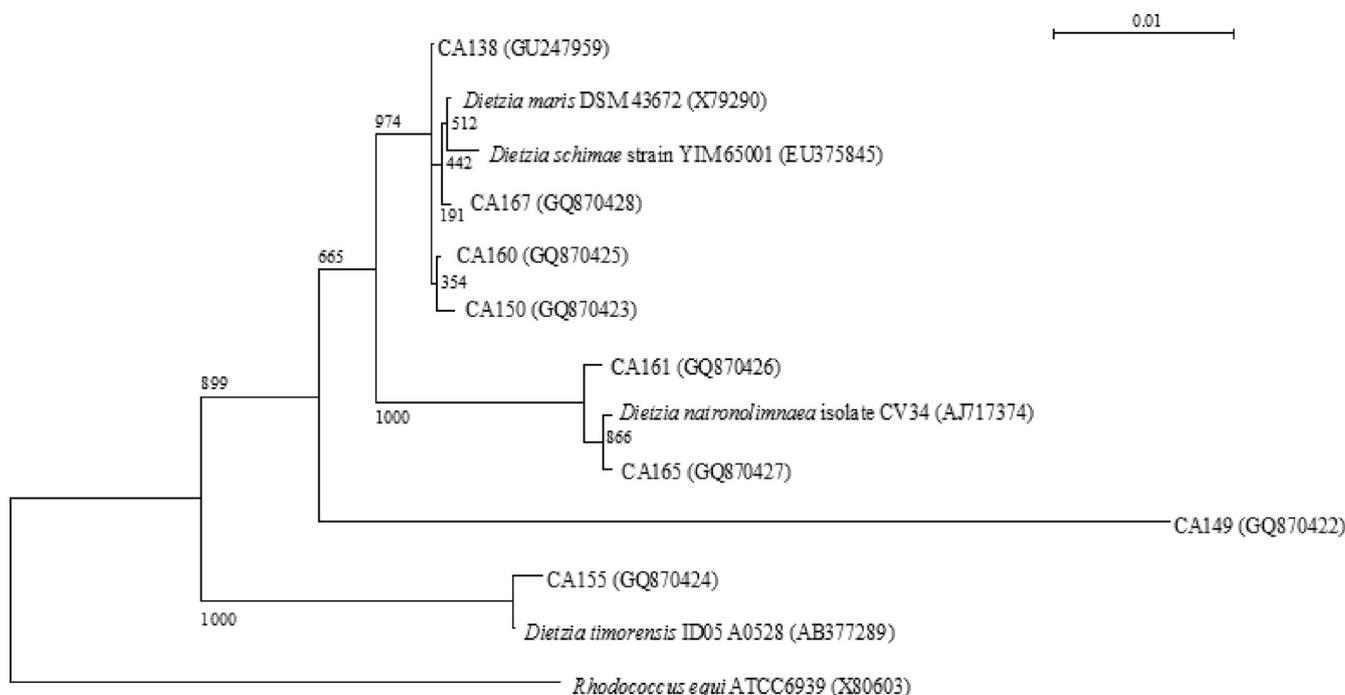


FIG. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships among isolates identified as *Dietzia* and representative strains of *Dietzia* and *R. equi*. Bar, 1% sequence variation. The bifurcations are supported by the indicated bootstrap values.

of mycobacteria, nocardiae, and other aerobic actinomycetes (7) were followed. The antibiotics recommended by the CLSI, including amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, trimethoprim-sulfamethoxazole (cotrimoxazole), imipenem, linezolid, minocycline, and tobramycin, were tested. Ampicillin and vancomycin were also tested. *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 served as controls. Resistance or susceptibility to the antibiotics was interpreted as suggested by the CLSI standards (7). The results of susceptibility tests are given in Table 2. The eight *Dietzia* isolates and ATCC 616106, a collection strain representative of *D. maris*, were susceptible to all antibiotics tested. *D. maris* has been reported previously to be susceptible to aztreonam, ciprofloxacin, mezlocillin, oxacillin, penicillin G,

perfloracin, and ticarcillin and to be resistant to sulfamethoxazole by disk diffusion testing (4).

Our findings show that discrimination between *R. equi* and *Dietzia* species is problematic in the clinical microbiology laboratory and that in the absence of clear-cut markers, 16S rRNA gene sequencing is required for accurate identification of isolates of these species. Species-level identification based on the full 16S rRNA gene sequence is considered to be the "gold standard" for actinomycetes (17), but results must be treated with caution for recently established and grossly undercharacterized genera, such as *Dietzia* (13). In our case, eight isolates erroneously identified as *R. equi* could be recognized as members of the genus *Dietzia* and seven of them could be identified at the species level.

TABLE 2. Antibiotic susceptibilities of the eight *Dietzia* isolates identified in this study

Isolate	MIC <sup>a</sup> (μg/ml) of:											
	AMK	AMC	AMP	CRO	CIP	CLR	COT	IPM	LZD	MIN	TOB	VAN
<i>D. maris</i> isolate CA138	0.25	0.016	0.047	0.06	0.032	0.016	1	0.125	0.125	0.064	0.19	0.19
<i>Dietzia</i> sp. isolate CA149	0.50	0.016	0.500	0.750	0.016	<0.016	0.094	0.064	0.250	0.032	0.500	0.500
<i>D. maris</i> isolate CA150	0.75	0.032	0.250	0.750	0.032	<0.016	0.094	0.047	0.190	0.047	0.750	0.125
<i>D. maris</i> isolate CA160	0.50	0.032	0.250	0.250	0.047	<0.016	0.032	0.064	0.380	0.125	0.190	0.500
<i>D. maris</i> isolate CA167	0.125	<0.016	0.047	0.750	0.008	<0.016	0.094	<0.002	0.016	<0.016	0.094	0.190
<i>D. natronolimnaea</i> isolate CA161	1.00	0.047	0.380	0.500	0.023	0.047	0.5	0.032	0.500	0.250	1.500	0.125
<i>D. natronolimnaea</i> isolate CA165	0.50	<0.016	0.250	0.750	0.012	0.250	0.19	0.032	0.190	0.023	0.500	<0.016
<i>D. timorensis</i> isolate CA155	0.125	0.016	0.750	0.750	0.032	0.120	0.25	0.064	0.032	0.064	0.023	0.500
<i>R. equi</i> ATCC 6939	2.00	0.016	0.380	0.094	0.047	<0.016	0.064	0.012	0.190	0.064	0.125	0.750
<i>D. maris</i> ATCC 616106	0.064	<0.016	0.023	0.032	0.006	<0.016	0.016	0.750	0.125	0.500	0.190	0.250

<sup>a</sup> The MICs were determined by the Etest method. The medium used was Mueller-Hinton agar supplemented with sheep blood. AMK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CRO, ceftriaxone; CIP, ciprofloxacin; CLR, clarithromycin; COT, cotrimoxazole; IPM, imipenem; LZD, linezolid; MIN, minocycline; TOB, tobramycin; VAN, vancomycin. *R. equi* ATCC 6939 and *D. maris* ATCC 616106 were included as reference strains.

*Dietzia* bacteria appear to be widely distributed in the environment, and reports of isolates from clinical material are increasing. *D. maris* was detected in skin samples from healthy subjects in a recent characterization of the skin microbiota using a 16S rRNA gene-based comprehensive analysis (8), suggesting that it may be a normal human commensal. Our finding of species of *Dietzia*, initially wrongly identified as *R. equi*, in human clinical specimens highlights the potential importance of members of this newly defined bacterial genus as opportunistic human pathogens and the need for accurate methods to ensure their correct identification in the clinical setting.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences from the eight *Dietzia* isolates were deposited in GenBank under accession numbers GU247959 and GQ870422 to GQ870428.

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