Characterization of *Corynebacterium* species in macaques

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Bacteria of the genus *Corynebacterium* are important primary and opportunistic pathogens. Many are zoonotic agents. In this report, phenotypic (API Coryne analysis), genetic (*rpoB* and 16S rRNA gene sequencing), and physical methods (MS) were used to distinguish the closely related diphtheroid species *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, and to definitively diagnose *Corynebacterium renale* from cephalic implants of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques used in cognitive neuroscience research. Throat and cephalic implant cultures yielded 85 isolates from 43 macaques. Identification by API Coryne yielded *C. ulcerans* ($n=74$), *Corynebacterium pseudotuberculosis* ($n=2$), *C. renale* or most closely related to *C. renale* ($n=3$), and commensals and opportunists ($n=6$). The two isolates identified as *C. pseudotuberculosis* by API Coryne required genetic and MS analysis for accurate characterization as *C. ulcerans*. Of three isolates identified as *C. renale* by 16S rRNA gene sequencing, only one could be confirmed as such by API Coryne, *rpoB* gene sequencing and MS. This study emphasizes the importance of adjunct methods in identification of coryneforms and is the first isolation of *C. renale* from cephalic implants in macaques.

INTRODUCTION

Reports of coryneform bacteria in the literature are becoming more prevalent, most notably due to the taxonomic changes in phylogenetic groupings (Funke *et al.*, 1997) and an increase in the number of invasive treatments and procedures performed on immunocompromised patients. The genus *Corynebacterium* is one of the largest in the coryneform group of bacteria, containing more than 60 species, 40 which are medically relevant (Funke *et al.*, 1997; Khamis *et al.*, 2005). They are Gram-positive, non-motile, facultative anaerobes, characterized as having the appearance of straight or slightly curved slender rods with tapered or clubbed ends (Funke *et al.*, 1997). *Corynebacterium* species cause opportunistic infections in both humans and domestic animals. *Corynebacterium diphtheriae*, the most widely known bacterium in the genus, is the causative agent of human diphtheria, a highly contagious upper respiratory tract infection which is still implicated in outbreaks worldwide. *C. diphtheriae* also causes cutaneous infections, endocarditis, septicaemia and osteomyelitis (Aubel *et al.*, 1997). Hall *et al.* (2010) recently identified a novel *C. diphtheriae* isolate from the ears of two domestic cats in West Virginia, but found no evidence of zoonotic transmission.

The non-diphtheroid species, specifically *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, have been shown to produce a variety of both animal and human diseases, and are important zoonotic pathogens. *C. pseudotuberculosis* is best known as the causative agent of caseous lymphadenitis in ruminants and ulcerative lymphangitis in horses (Dorella *et al.*, 2006; Pacheco *et al.*, 2007). Human infections with these bacteria, although uncommon, have been documented, with the majority of cases resulting from occupational exposure (Dorella *et al.*, 2006). *C. ulcerans* was first isolated in 1926 by Gilbert and Stewart from human

Abbreviation: MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight MS.

pharyngeal cultures (Funke *et al.*, 1997). It has been reported as a cause of pharyngitis, granulomatous pneumonia, and less commonly diphtheria, in humans (Funke *et al.*, 1997). The traditional association of *C. ulcerans* infection of humans is zoonotic transmission from cattle or the consumption of raw milk from infected cattle (Lartigue *et al.*, 2005). It is now the most common cause of diphtheria in the UK (Wagner *et al.*, 2010). Recent literature also suggests that *C. ulcerans* isolated from domestic pigs (Schuhegger *et al.*, 2009), domestic cats (De Zoysa *et al.*, 2005) and dogs (Lartigue *et al.*, 2005; Katsukawa *et al.*, 2009) may be a reservoir for human infection. We have previously described *C. ulcerans* from a case of mastitis in a bonnet macaque and as a frequent contaminant of cephalic implants from macaques used in cognitive neuroscience (Fox & Frost, 1974; Bergin *et al.*, 2000).

Corynebacterium species are potentially zoonotic, so rapid and accurate discrimination of these organisms is crucial. In diagnostic laboratories, Analytical Profiling Index (API) is a common, rapid and inexpensive method used to identify closely related bacteria. For *Corynebacterium* species, the API Coryne test is fairly reliable, citing 97.71 % of the strains being correctly identified (with or without supplementary tests), 1.28 % of the strains not identified, and 1.01 % of the strains misidentified [personal communication from bioMérieux (or http://www.biomerieux.com/servlet/srt/bio/portail/home)]. However, the test can be subjective, can only detect known coryneforms, requires bacterial suspensions of adequate turbidity, and may not discriminate between closely related species.

Historically, the 16S rRNA gene sequence has been considered the gold standard for determination of the phylogenetic relationship among bacteria. Unfortunately, the 16S rRNA gene sometimes lacks the high intra-genus polymorphism that is needed for precise taxonomic analysis and species discrimination (Khamis *et al.*, 2004). The percentage similarity in the 16S rRNA gene sequence between *C. diphtheriae* and *C. ulcerans* has been reported to be 98.5; between *C. diphtheriae* and *C. pseudotuberculosis*, 98.5; and between *C. ulcerans* and *C. pseudotuberculosis*, 99.7 % (Khamis *et al.*, 2004).The RNA polymerase beta subunit-encoding gene (*rpoB*) is a universal gene that has been used in the phylogenetic analysis of a variety of bacteria, and has been highly beneficial in distinguishing among closely related isolates (Adékambi *et al.*, 2009). This method has been used in the past to demonstrate the variability among species, isolates, serotypes and biotypes for *Escherichia coli*, *Salmonella enterica*, *Vibrio cholerae* and *Haemophilus influenzae* (Adékambi *et al.*, 2009). Furthermore, sequencing of the hypervariable region of the *rpoB* gene has allowed for the identification of unknown isolates in the bacterial orders Aquificales and Rhizobiales (Adékambi *et al.*, 2009). Khamis *et al.* (2004) obtained almost complete *rpoB* sequences of several isolates of *Corynebacterium* species and identified an area with a high degree of polymorphism (hypervariable region) for subsequent primer design. With complete sequencing of the *rpoB* gene, the percentage similarity between *C. ulcerans* and *C. pseudotuberculosis* drops to 93.6 %, and the percentage similarity between *C. diphtheriae* and *C. ulcerans* drops to 86 % (Khamis *et al.*, 2004). The corresponding similarity between and among these species using 16S rRNA gene sequence analysis was over 98.5 %. They also demonstrate that two *Corynebacterium* isolates belong to the same species if they show 95 % or greater similarity, and argue that by using the *rpoB* gene sequencing analysis, a more discriminatory characterization of isolates can be obtained (Khamis *et al.*, 2005).

To further define the status of *Corynebacterium* species in macaques housed in our vivarium, we have now collected samples from the cephalic implants and oropharynges of all implanted non-human primates. We initially characterized coryneform isolates from non-human primates using API testing and 16S rRNA gene sequencing analysis; however, discrepancies among selected samples were discovered. Isolates identified as *C. pseudotuberculosis* on API testing were characterized as *C. ulcerans* by 16S rRNA analysis. As both analytical methods used for confirming *Corynebacterium* species have potential limitations, we assessed two additional methods of identification, *rpoB* gene sequencing analysis and matrix-assisted laser desorption/ionization-time of flight MS (MALDI-TOF MS), in an effort to resolve discordant results and provide diagnostic adjuncts to 16S rRNA sequencing for definitive diagnosis.

METHODS

Animals. Macaques were singly or pair housed in stainless steel quadrangles of four units with individual cage dimensions of $31 \times 29 \times 64$ inches ($78.7 \times 73.7 \times 163$ cm). Animals were fed *ad libitum* with commercial primate chow (Lab Diet 5038, PMI Nutrition International) and daily fruits, vegetables and treats. All animals were used in cognitive neuroscience research and, in accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocols, had periodic limited restriction of access to water. Animals that were off-study had water available *ad libitum*. Macaques were housed in an AAALAC International-accredited animal facility with 10–15 complete air changes an hour, a 12 h light : dark cycle, and temperature and humidity levels of 72–78 °F (22–26 °C) and 30–70 %, respectively.

Microbiology. Isolates were cultured from oropharynx and cephalic implants of rhesus monkeys (*Macaca mulatta*, 32 males and 9 females) and cynomolgus monkeys (*Macaca fascicularis*, $n=2$ males) over a period of approximately 4 years. An individual sterile bacterial transport culturette (Venturi Transystem Transport Swab, Copan Diagnostics) was swabbed across the oropharynx, interior of the cephalic recording chamber, or skin/implant interface of implants on each animal. Swabs were streaked onto 5 % sheep blood, MacConkey and chocolate agar plates as previously described (Bergin *et al.*, 2000). Swabs were also placed in trypticase soy broth for enrichment; plates and broth were incubated for 18–24 h at 37 °C and 5 % CO_2. A blood agar plate was also incubated at room temperature to prevent overgrowth by non-coryneforms. Aliquots of broth were subsequently subcultured onto blood, MacConkey and chocolate agar plates (Bergin *et al.*, 2000).

Pure cultures were obtained by restreaking single colonies onto blood agar. These were subsequently characterized by Gram staining and

coryneforms were then speciated using the API Coryne strip system (bioMérieux) according to instructions from the manufacturer. Plates with optimum bacterial growth were used for DNA extraction and collected in freeze medium (*Brucella* broth and 20 %, v/v, glycerol) for storage at −70 °C. Coryneforms identified as *C. pseudotuberculosis* or *Corynebacterium renale* were tested by a second laboratory [Centers for Disease Control and Prevention (CDC), Atlanta, GA] using the API Coryne strip system.

DNA extraction. The High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) was used to extract DNA from bacterial pellets, as described previously (Fox *et al.*, 2009).

16S rRNA gene sequencing. The sequences of the 16S rRNA genes of 17 selected isolates were performed as described by Dewhirst *et al.* (2010). These isolates had been presumptively identified by our laboratory using the API Coryne strip system as *C. pseudotuberculosis* (n=14) or *C. renale* (n=3). Briefly, primers F24 (positions 9–27 in the forward direction) and F25 (positions 1525–1541 in the reverse direction) were used to amplify the 16S rRNA genes. The PCR product was concentrated and purified with QIAquick PCR purification kits (Qiagen). Purified DNA was sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit) on an ABI 3100 genetic analyser (Applied Biosystems). The sequencing primers (Dewhirst *et al.*, 2010) were used in quarter-dye reactions, according to the manufacturer's instructions. The 16S rRNA gene sequences were entered into RNA, a program for analysis of 16S rRNA gene data, and were aligned as described elsewhere (Jukes & Cantor, 1969; Paster & Dewhirst, 1988). The aligned sequences were exported and analysed using MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrapping was performed with 1000 replicates.

***rpoB* gene sequencing.** The sequences of the partial *rpoB* gene were obtained for the 17 isolates originally identified as *C. pseudotuberculosis* or *C. renale* by API Coryne. The conserved primers C2700F and C3130R from the *rpoB* gene were used to amplify the PCR products, as described by Khamis *et al.* (2004). We detected PCR products of a size similar to those reported by Khamis *et al.* (2004). The amplicons were purified and directly sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The sequences were aligned using CLUSTAL W and analysed using MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). Bootstrapping was performed with 1000 replicates.

***tox* gene.** PCR amplification of the diphtheria toxin (*tox*) gene was performed on all isolates and used specific primers against the A and B subunits, as described by Schuhegger *et al.* (2008). NCTC 10648 was used as the positive control and NCTC 10356 as the negative control.

***pld* gene.** PCR amplification of the phospholipase D (*pld*) gene was performed on the 17 isolates originally identified as *C. pseudotuberculosis* or *C. renale* by API Coryne. The primers used were derived from the *C. ulcerans* (*pld*) sequence (GenBank accession no. L16585). The sequence of the forward primer (PLD-1) was 5′-TGTTTC-ACATGACGCAGCTT-3′; the sequence of the reverse primer (PLD-2) was 5′-AAGATCATTCCGTCTACATGA-3′. Reagents without DNA were used as a negative control; 720 bp PCR products from the isolates were sequenced and confirmed to have 98–99 % homology with the *C. ulcerans pld* gene. Conditions were as described previously (Bergin *et al.*, 2000).

Elek toxigenicity test. All coryneform isolates were tested for the production of diphtheria toxin using the conventional Elek assay as described by Engler *et al.* (1997). Briefly, two test strains and three control strains (positive control: NCTC 10648, *C. diphtheriae gravis*; negative control: NCTC 10356, *C. diphtheriae belfanti*; weak positive control: NCTC 3984, *C. diphtheriae gravis*) were inoculated in straight lines across each plate of Elek medium. A filter paper strip containing 500 IU diphtheria antitoxin ml^{-1} was placed across the agar surface and perpendicular to the inoculation lines. Plates were incubated in air at 37 °C for 48 h and examined for precipitin lines after 24 and 48 h. Non-toxigenic strains produced no precipitin lines.

MALDI-TOF MS. The 17 isolates originally identified by API Coryne as *C. pseudotuberculosis* or *C. renale* were analysed by MALDI-TOF MS to determine their protein patterns. The data were searched within the Bruker BioTyper database using the Bruker BioTyper software (Bruker Daltonics). Isolates were prepared using the ethanol/formic acid extraction procedure recommended by the manufacturer, spotted on the target, allowed to dry, and overlaid with alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker, 255344). The MALDI-TOF MS analysis was performed using a Bruker Ultraflex III mass spectrometer operated in positive linear mode. The instrument was calibrated before the analysis using Bruker bacterial test standards (255343).

RESULTS

Microbiology

Eighty-five isolates were obtained from 43 macaques: eight from throat cultures and 77 from implants. The isolates were identified by API Coryne strips as *C. ulcerans* (n=60), *C. pseudotuberculosis* (n=14) and *C. renale* (n=3). Of the isolates obtained from throat cultures, one (07-2012) was identified as *C. renale* and the others were identified as *C. ulcerans*. Isolates identified as *C. pseudotuberculosis* and *C. renale* (n=17) at our laboratory (MIT) were evaluated by a separate laboratory (CDC) using the same method. Twelve of 14 were interpreted to be *C. ulcerans* (likelihood 99.8 %), 2/14 were interpreted to be *C. pseudotuberculosis* (likelihood greater than 97.6 %), and 1/3 closely matched *C. renale* (likelihood 99.8 %). The remaining two isolates originally classified as *C. renale* produced API codes that were less closely matched but which were certainly members of the genus *Corynebacterium* (Table 1).

A number of additional coryneforms were isolated from throat swabs and identified by API Coryne as *Corynebacterium propinquum* (n=4), *Corynebacterium auris* (n=1) and *Corynebacterium minitissimum* (n=1). Two isolates of *Corynebacterium striatum/amycolatum* were isolated from cephalic implants. These organisms were not further characterized.

16S rRNA gene and *rpoB* gene sequencing

Full-length 16S rRNA sequences were obtained for the 17 strains identified by API at the second laboratory as *C. ulcerans*, *C. pseudotuberculosis* or *C. renale*. A phylogenetic tree for representative isolates is shown in Fig. 1. All of the *C. ulcerans* or *C. pseudotuberculosis* strains were identified as closest to the sequence for the type strain of *C. ulcerans*,

Table 1. Identification of isolates using API Coryne, MALDI-TOF MS and gene sequencing modalities

Percentages after the API identification refer to confidence limits. Percentages after the gene sequencing results refer to percentage identities with respect to a reference strain.

Accession	CDC API code	CDC API interpretation	CDC MALDI-TOF MS	16S rRNA	*rpoB*
05-1536	0110326	*C. ulcerans* (85.6 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
06-0265	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.5 %)	*C. ulcerans* (99.5 %)
07-0051	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.7 %)	*C. ulcerans* (99.5 %)
06-0572	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-1734	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-1845	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.7 %)	*C. ulcerans* (99.5 %)
07-1845	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.7 %)	*C. ulcerans* (99.5 %)
07-0690	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.7 %)	*C. ulcerans* (99.5 %)
07-1694	2001304	*C. renale* group (90.1 %, doubtful profile)	*Aromatoleum aromaticum*	*C. renale* (98.8 %)	*C. renale* (92.3 %)
07-2012	2001304	*C. renale* group (90.1 %, doubtful profile)	No identification	*C. renale* (98.8 %)	*C. renale* (92.3 %)
07-2044	2201304	*C. renale* group (99.8 %)	*C. renale*	*C. renale* (99.2 %)	*C. renale* (98.5 %)
07-2017	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-2027	0101304	*C. pseudotuberculosis* (97.6 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-7334	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-7343	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
07-7343	0111304	*C. pseudotuberculosis* (99.3 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)
08-0538	0111326	*C. ulcerans* (99.7 %)	*C. ulcerans*	*C. ulcerans* (99.6 %)	*C. ulcerans* (99.5 %)

but also very close to *C. pseudotuberculosis*. By BLASTN analysis, the 16S rRNA sequences of the three remaining strains were closest to *C. renale*. Strain 07-2044 had 99.2 % sequence similarity and strains 07-1694 and 07-2012 had 98.8 % similarity to *C. renale*. The lack of clear species separation by 16S rRNA for some taxa within the genus *Corynebacterium* is apparent in the tree. The partial *rpoB* gene sequences obtained for these strains and the phylogenetic tree for representative isolates is shown in Fig. 2. The strains identified by API as *C. ulcerans* or *C. pseudotuberculosis* all cluster with the type strain of *C. ulcerans* (0.5 % divergent), and *C. pseudotuberculosis* is well resolved as a separate taxon (8.5 % divergent). The *rpoB* sequence for strain 07-2044 is highly similar to that of *C. renale* (1.5 % divergent), while those of strains 07-1694 and 07-2012 diverged by 8.2 % from *C. renale*. The latter two isolates are therefore unlikely to be *C. renale*, as the limit for species identity with *rpoB* is 95 % and both genetic and physical methods indicate another species. *rpoB* gene sequencing in this instance was in general agreement with the phenotypic characterization of the API Coryne code generated at the second laboratory (90.1 % likelihood). It is clear from comparing the 16S rRNA and the *rpoB* trees that *rpoB* has superior ability to resolve the phylogeny of *Corynebacterium* spp.

Elek toxigenicity test and *tox* gene

Neither toxin activity nor the *tox* gene was detected by the Elek test and PCR amplification, respectively.

pld gene

Sixteen of 17 isolates were positive by *pld* PCR.

MALDI-TOF MS

The 14 isolates identified as *C. ulcerans* or *C. pseudotuberculosis* by API were identified in the CDC MALDI-TOF MS database as *C. ulcerans*. Strain 07-2044, identified as *C. renale* by API, was similarly identified by CDC MALDI-TOF MS. The two novel *Corynebacterium* spp. strains were either not identified or misidentified (as *Aromatolecum aromaticum*, an unrelated organism from the phylum Proteobacteria), as the novel organism is not in the CDC MALDI-TOF MS database.

DISCUSSION

In this study, we used phenotypic, biochemical, genetic and physical methods for identification of coryneforms from clinical specimens. We initially used API to identify *C. ulcerans*, *C. renale* and *C. pseudotuberculosis* from the cephalic implants and oropharynx of macaques. *C ulcerans* had been previously identified in our macaque population; the isolation of two additional species of Corynebacteria motivated efforts to confirm the identity of these latter isolates. Corynebacteria that were not identified as *C. ulcerans* were then evaluated by API at CDC, and some discordant results were generated. These 17 isolates were subjected to additional analysis (MALDI-TOF MS, 16S

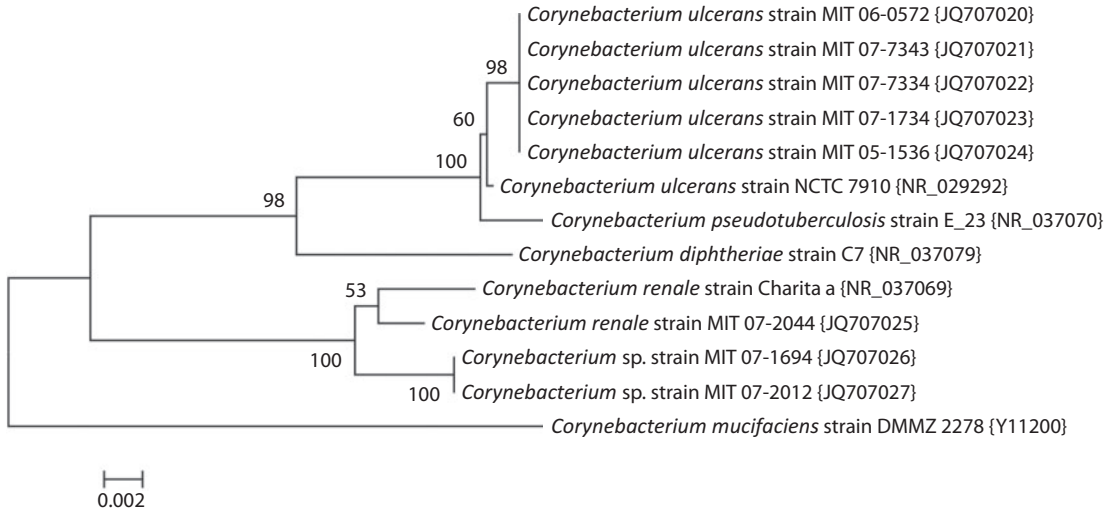

Fig. 1. Evolutionary relationships of *Corynebacterium* based on 16S rRNA sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length=0.09719690 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 13 nt sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1494 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

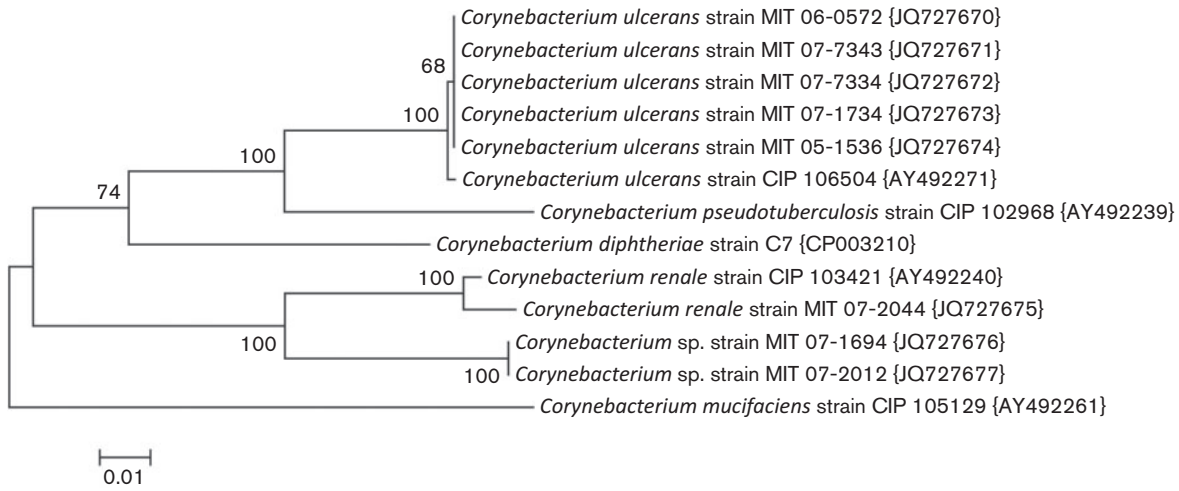

Fig. 2. Evolutionary relationships of *Corynebacterium* based on the *rpoB* sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length=0.44347215 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes–Cantor method (Jukes & Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 13 nt sequences. All ambiguous positions were removed for each sequence pair. There were a total of 369 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

rRNA and *rpoB* gene sequencing), and 14 isolates were confirmed by all three methods to be *C. ulcerans*. API testing is a rapid method frequently used in diagnostic settings, but may not be adequately discriminatory to distinguish between closely related *Corynebacterium* species. This conclusion is in agreement with Contzen *et al.* (2011), who found that API could not identify unequivocally two isolates of *C. ulcerans* from wild boars. API interpretations can be subjective or require ancillary tests; moreover, expression of phenotypic characters may vary depending upon environmental pressures such as antibiotic administration (Drancourt *et al.*, 2000). The critical difference between *C. ulcerans* and *C. pseudotuberculosis* on API Coryne is glycogen fermentation. Positive tests in maltose and glycogen fermentation generate an API code of 0111326, signifying 99.7 % confidence in an identification of *C. ulcerans*. Negative tests for fermentation of these substrates generates a code of 0111304 and 99.3 % confidence in a diagnosis of *C. pseudotuberculosis*. Ancillary chemotaxonomic methods for distinguishing between these organisms include trehalose fermentation (*C. ulcerans* +; *C. pseudotuberculosis* −), amylase activity (*C. ulcerans* +; *C. pseudotuberculosis* −), and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamide hydrolysis (60 % of *C. ulcerans* isolates +; *C. pseudotuberculosis* −) (Contzen *et al.*, 2011; Kämpfer, 1992). Contzen *et al.* (2011) demonstrated that addition of trehalose fermentation to the reactions contained in API Coryne allowed 100 % discrimination of isolates of *C. pseudotuberculosis* and *C. ulcerans* from multiple host species. More variable characteristics that have been noted include alkaline phosphatase (*C. ulcerans* +; *C. pseudotuberculosis* variable but often −) and nitrate reduction (*C. ulcerans* −; *C. pseudotuberculosis* variable) (Funke *et al.*, 1997). Cellular polar lipid profiles and fatty acid profiles can also be useful in distinguishing closely related species (Frischmann *et al.*, 2011).

MALDI-TOF MS uses the protein composition of abundant protein species in bacterial cells for identification of isolates. The spectrum generated by the MS process is compared with reference spectra and a specific identification can be made if the isolate is a species included in the reference database. In a study of 116 *Corynebacterium* species isolates submitted to the German Consiliary Laboratory, MALDI-TOF MS showed agreement with *rpoB* gene sequencing for 115 of 116 isolates (99.1 %) (Konrad *et al.*, 2010). The only isolate for which MALDI-TOF MS was accurate only to the genus level was one identified by *rpoB* as *Corynebacterium tuberculostearicum*. In contrast, API Coryne results were ambiguous at the species level for 12 isolates (11.2 %). API Coryne did, however, identify correctly and congruently all isolates of the coryneforms *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*. MALDI-TOF MS can complement traditional phenotypic and taxonomic methods in a high-throughput fashion with little sample preparation if dealing with organisms in the reference database.

As 16S rRNA gene sequencing has low intra-genus polymorphism for *Corynebacterium* species, we evaluated selected isolates by *rpoB* gene sequencing. Studies by Khamis *et al.* (2004, 2005) had demonstrated that high degrees of similarity in 16S rRNA gene sequencing among isolates did not correlate with degree of similarity in *rpoB* gene sequencing. They further showed that the hypervariable region sequence of between 434 and 452 base pairs of the *rpoB* gene was superior to 16S rRNA gene sequencing in discrimination of closely related species. In this study, complete gene sequencing of the 16S rRNA gene was concordant with that of the hypervariable region of *rpoB*. In two of our 74 isolates (2.7 %) identified by API Coryne as either *C. ulcerans* or *C. pseudotuberculosis*, molecular genotyping was required for accurate identification.

In macaques, skin erosion and necrosis, as well as the generation of exuberant granulation tissue at the skin–implant interface, are potential clinical sequelae of cephalic implant placement. *C. ulcerans* may have been associated with bilateral chronic skin ulcers in a Brazilian woman with pulmonary infection from whom the organism was isolated in a bronchoalveolar lavage (BAL) sample (Mattos-Guaraldi *et al.*, 2008). While *C. ulcerans* was not cultured specifically from the ulcers, the lesions regressed during antimicrobial therapy directed at the BAL isolate. In another report, a 71-year-old man with chronic non-healing ulcers of the right leg was diagnosed with toxigenic *C. ulcerans* (Wagner *et al.*, 2001). The lesions resembled those of cutaneous diphtheria, a well-recognized clinical entity caused by non-toxigenic *C. diphtheriae* (Lowe *et al.*, 2011). Similar skin lesions have been reported in the *C. pseudotuberculosis*-associated diseases, oedematous skin disease of buffalo and ulcerative lymphangitis of horses (Selim, 2001). Clinical features of infection by *C. ulcerans* and highly related organisms suggest a potential aetiological role for *C. ulcerans* in these outcomes in macaques. The prevalence of mixed infections in affected macaques and the lack of demonstration of PLD toxin elaboration from the isolates reported here make unequivocal association difficult, however.

We report for the first time the isolation of *C. renale* from the cephalic implant of a macaque, a finding confirmed by *rpoB* gene sequencing analysis. The performance of the identification modalities regarding the three putative *C. renale* isolates reflects the difficulty in definitive diagnosis of some isolates. Two of the three isolates did not reach the 95 % identity threshold recommended by Khamis *et al.* (2005) for species identity in *rpoB* gene sequencing. API Coryne and MALDI-TOF MS analysis were in concordance with that of *rpoB*, while both disagreed with the results of 16S rRNA gene sequencing. *C. renale* is the most common causative agent of urogenital disease in ruminants (Funke *et al.*, 1997) and has been previously identified in laboratory animals. Stevens *et al.* (2007) reported the isolation of the organism from the urinary bladder of a rhesus monkey with necrohaemorrhagic cystitis. This finding, however, was based solely on microbial culture and Gram staining, without confirmation by molecular methods. There have been cases of spontaneous urinary calculi in young

laboratory rats reportedly caused by *C. renale* (Osanai *et al.*, 1994; Takahashi *et al.*, 1995). The clinical impact of *C. renale* on cephalic implants of macaques is uncertain.

In this study, we have isolated an additional species of *Corynebacterium* from cephalic implants of macaques, affirmed the difficulty of distinguishing among closely related coryneforms, and demonstrated the use of *rpoB* gene sequencing and MALDI-TOF MS as discriminatory tests for identification of closely related isolates. Although the 16S rRNA and *rpoB* gene sequencing methods yielded identical results, *rpoB* did not require sequencing of the entire gene. MALDI-TOF MS analysis, though fast and accurate for species in its database, is currently restricted to large diagnostic centres and reference laboratories. Finally, Pacheco and co-workers, working with clinical isolates of *C. pseudotuberculosis* from small ruminants with caseous lymphadenitis, were able to distinguish between *C. pseudotuberculosis* and *C. ulcerans* using a multiplex PCR capable of detecting the 16S rRNA, *rpoB* and *pld* genes (Pacheco *et al.*, 2007).

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