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TITLE PAGE

Characterization of *Corynebacterium* species in macaques

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Running Title: *Corynebacterium* species in macaques

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ABSTRACT

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 (mass spectroscopy) were used to distinguish the closely related diphtheroid species *C. ulcerans* and *C. pseudotuberculosis*, and to definitively diagnose *C. 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), *C. 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 mass spectroscopic 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 mass spectroscopy. 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, septicemia, 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 *C. ulcerans* and *C. 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 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 United Kingdom (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 and 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 *. 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 intragenus polymorphism that

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is needed for precise taxonomic analysis for 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 (Adekambi 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 cholera*, and *Haemophilus influenzae* (Adekambi 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*. (Adekambi et al., 2009). Khamis et al. (2004) performed 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 (Khamis et al., 2004). 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 nonhuman primates. We initially characterized coryneform isolates from nonhuman 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 mass spectrometry (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 x 29 x 64 inches. Animals were fed *ad libitum* with commercial primate chow (Lab Diet 5038, PMI Nutrition International; Brentwood, MO) 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 hour light:dark cycle, and temperature and humidity levels of 72-78° F (27° 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 Inc.; Corona, CA) 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 hrs at 37° C and 5 % CO₂. A blood agar plate was also incubated at room temperature to prevent overgrowth by non-coryneforms. Aliquots of broth were subsequently sub-cultured onto blood, MacConkey and chocolate agar plates (Bergin et al., 2000).

Pure cultures were obtained by re-streaking single colonies onto blood agar. These were subsequently characterized by Gram staining and coryneforms were then speciated using the API Coryne strip system (bioMérieux; Durham, NC) 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% glycerol) for storage at -70° C. Coryneforms identified as *C. pseudotuberculosis* or *C. renale* were tested by a second laboratory (The Centers for Disease Control and Prevention (CDC), Atlanta Georgia) using the API Coryne strip system.

DNA Extraction. The high Pure PCR Template Preparation kit (Roche Molecular Biochemicals; Indianapolis, IN) 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 select 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 to 27 in the forward direction) and F25 (positions 1525 to 1541 in the reverse direction) were used to amplify the 16S rRNA genes. The PCR product was concentrated and purified with QIAquickPCR purification kits (Qiagen; Valencia, CA). Purified DNA was sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator cycle sequencing kit) on an ABI 3100 genetic analyzer (Applied Biosystems; Foster City, CA). 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 previously (Jukes and Cantor, 1969; Paster and Dewhirst, 1988). The aligned sequences were exported and analyzed 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.

***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 *rpoB* gene were used to amplify the polymerase chain reaction (PCR) products as previously described (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, Foster City, CA). The sequences were aligned using Clustal W and analyzed 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 previously described (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 L16585). The sequence of the forward primer (PLD-1) was 5'TGTTTCACATGACGCAGCTT-3'; the sequence of the reverse primer (PLD-2) was 5'AAGATCATTCGCTCTACATGA-3'. [Reagents without DNA was 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 previously (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/ml diphtheria antitoxin 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 analyzed by MALDI-TOF-MS to determine their protein patterns. The data was searched within the Bruker BioTyper database using the Bruker BioTyper software (Bruker Daltonics; Fremont, CA). Isolates were prepared using the ethanol/formic acid extraction procedure recommended by the manufacturer, spotted on target, allowed to dry, and overlaid with alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker, 255344). The MALDI-TOF-MS analysis was performed using 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: 8 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 2 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 *C. propinquum* (n=4), *C. auris* (n=1), and *C. minitissimum* (n=1). Two isolates of *C. 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*, 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 taxa (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.

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

202 **MALDI-TOF-MS:** The 14 isolates identified as *C. ulcerans* or *C. pseudotuberculosis* by API were identified in the CDC

203 MALDI-TOF-MS database as *C. ulcerans*. Strain 07-2044, identified as *C. renale* by API was similarly identified by

204 CDC MALDI-TOF-MS. The two novel *Corynebacterium* spp. strains were either not identified or misidentified (as

205 *Aromatolecum aromaticum*, an unrelated organism from the phylum Proteobacteria) as the novel organism is not in the

206 CDC MALDI-TOF-MS database.

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208

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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 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-MU-N-acetyl-B-D-glucosamide hydrolysis (60% of *C. ulcerans* isolates +; *C. pseudotuberculosis* -), (Contzen et al., 2011; Kampfer 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 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 profile and fatty acid profile can also be useful in distinguishing closely related species (Frischmann et al., 2011).

MALDI-TOF-MS uses protein composition of abundant protein species in bacterial cells for identification of isolates. The spectrum generated by the mass spectroscopy process is compared to 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 *C. 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.

245 As 16S rRNA gene sequencing has low intragenus polymorphism for *Corynebacterium* species, we evaluated select
246 isolates by *rpoB* gene sequencing. Studies by Khamis et al. (2004, 2005) had demonstrated that high degrees of similarity
247 in 16S rRNA gene sequencing among isolates did not correlate with degree of similarity in *rpoB* gene sequencing. They
248 further showed that the hypervariable region sequence of between 434-452 base pairs of the *rpoB* gene was superior to
249 16S rRNA gene sequencing in discrimination of closely related species. In the study reported here, complete gene
250 sequencing of 16S rRNA gene was concordant with that of the hypervariable region of *rpoB*. In 2 of our 74 isolates
251 (2.7%) identified by API Coryne as either *C. ulcerans* or *C. pseudotuberculosis*, molecular genotyping was required for
252 accurate identification.

253 In macaques, skin erosion and necrosis, as well as the generation of exuberant granulation tissue at the skin-implant
254 interface are potential clinical sequelae of cephalic implant placement. *C. ulcerans* may have been associated with
255 bilateral chronic skin ulcers in a Brazilian woman with pulmonary infection from whom the organism was isolated in a
256 bronchoalveolar lavage (BAL) sample (Mattos-Guaraldi et al., 2008). While *C. ulcerans* was not cultured specifically
257 from the ulcers, the lesions regressed during antimicrobial therapy directed at the BAL isolate. In another report, a 71
258 year old man with chronic non-healing ulcers of the right leg was diagnosed with toxigenic *C. ulcerans* (Wagner et al.,
259 2001). The lesions resembled those of cutaneous diphtheria, a well-recognized clinical entity caused by non-toxigenic *C.*
260 *diphtheriae* (Lowe et al., 2011). Similar skin lesions are reported in the *C. pseudotuberculosis*-associated diseases,
261 edematous skin disease of buffalo and ulcerative lymphangitis of horses (Selim, 2001). Clinical features of infection by
262 *C. ulcerans* and highly related organisms suggest a potential etiologic role for *C. ulcerans* in these outcomes in macaques.
263 The prevalence of mixed infections in affected macaques and the lack of demonstration of PLD toxin elaboration from
264 isolates reported here make unequivocal association difficult however.

265 We report for the first time the isolation of *C. renale* from the cephalic implant of a macaque, a finding confirmed by
266 *rpoB* gene sequencing analysis. The performance of the identification modalities regarding the three putative *C. renale*
267 isolates reflects the difficulty in definitive diagnosis of some isolates. Two of the three isolates did not reach the 95%
268 identity threshold recommended by Khamis et al. (2005) for species identity in *rpoB* gene sequencing. API Coryne and
269 MALDI-TOF-MS analysis were in concordance with that of *rpoB* while both disagreed with the results of 16S rRNA gene
270 sequencing. *C. renale* is the most common causative agent of urogenital disease in ruminants (Funke et al., 1997) and has
271 been previously identified in laboratory animals. Stevens et al. (2007) report the isolation of the organism from the
272 urinary bladder of a rhesus monkey with necrohemorrhagic cystitis. This finding was based solely on microbial culture
273 and Gram stain, without confirmation by molecular methods. There have been cases of spontaneous urinary calculi in
274 young laboratory rats reportedly caused by *C. renale* (Osanai et al., 1994; Takahashi et al., 1995). The clinical impact of
275 *C. renale* on cephalic implants of macaques is uncertain.

276 In this study, we have isolated an additional species of *Corynebacterium* from cephalic implants of macaques, affirm the
277 difficulty of distinguishing among closely related coryneforms, and demonstrate the use of the *rpoB* gene sequencing and
278 MALDI-TOF-MS as discriminatory tests for identification of closely-related isolates. Although the 16S rRNA and *rpoB*
279 gene sequencing methods yielded identical results, *rpoB* did not require sequencing the entire gene. MALDI-TOF-MS
280 analysis, though fast and accurate for species in its database, is currently restricted to large diagnostic centers and

reference laboratories. Finally, Pacheco et al., working with clinical isolates of *C. pseudotuberculosis* from small ruminants with caseous lymphadenitis, was 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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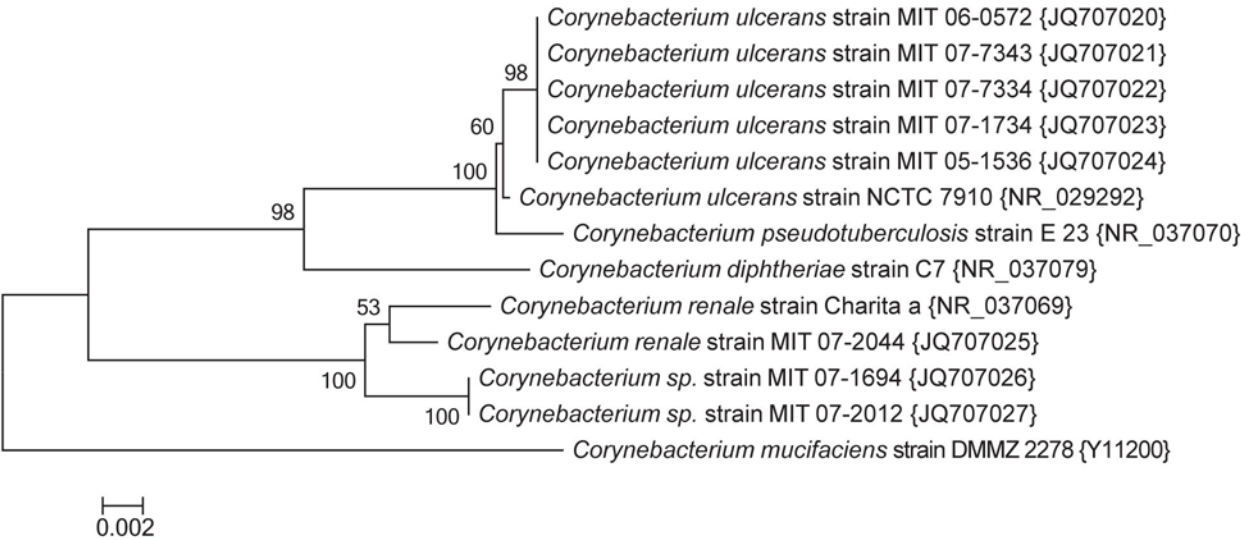


Figure. 1. Evolutionary relationships of *Corynebacterium* based on 16S rRNA sequences.

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 nucleotide 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).

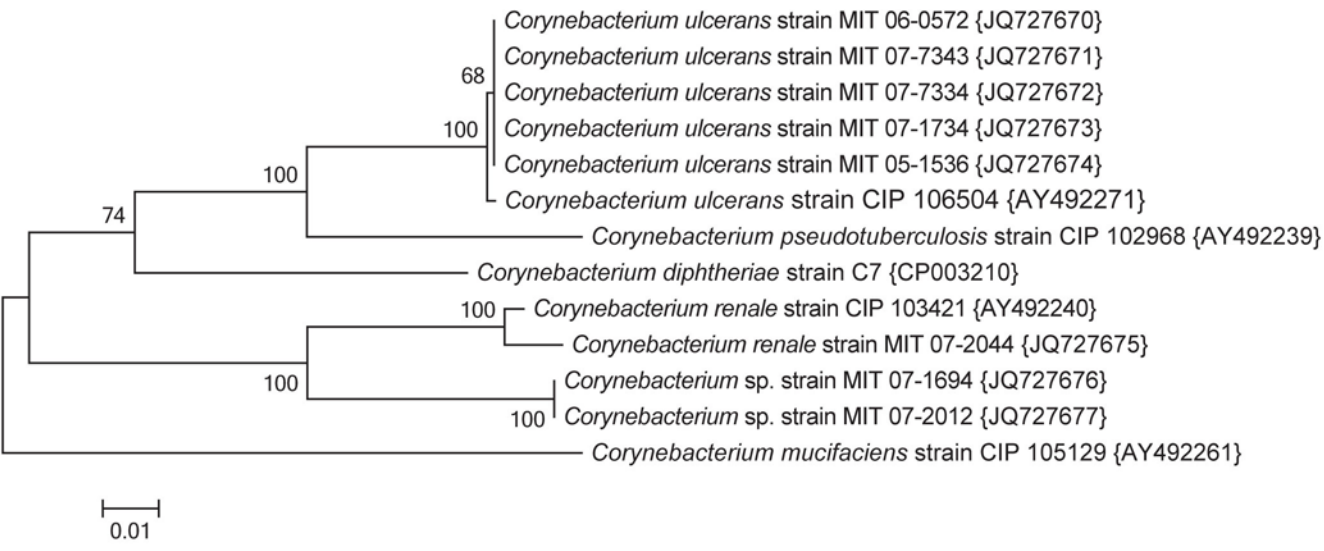


Figure 2. Evolutionary relationships of *Corynebacterium* based on the *rpoB* sequences.

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 nucleotide 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).

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 a reference strain.

Accession	CDC API	CDC API interpretation	CDC MALDI-TOF-MS	16S rRNA	rpoB
05-1536	0110326	<i>C. ulcerans</i> (85.6%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
06-0265	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.5%)	<i>C. ulcerans</i> (99.5%)
07-0051	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i> (99.7%)
06-0572	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-1734	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-1845	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i> (99.7%)
07-1845	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i> (99.7%)
07-0690	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i> (99.7%)
07-1694	2001304	<i>C. renale</i> group (90.1%, doubtful profile)	<i>Aromatoleum aromaticum</i>	<i>C. renale</i> (98.6%)	<i>C. renale</i> (98.6%)
07-2012	2001304	<i>C. renale</i> group (90.1%, doubtful profile)	no identification	<i>C. renale</i> (98.4%)	<i>C. renale</i> (98.4%)
07-2044	2201304	<i>C. renale</i> group (99.8%)	<i>C. renale</i>	<i>C. renale</i> (98.9%)	<i>C. renale</i> (98.9%)
07-2017	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-2027	0101304	<i>C. pseudotuberculosis</i> (97.6%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-7334	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-7343	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
07-7343	0111304	<i>C. pseudotuberculosis</i> (99.3%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)
08-0538	0111326	<i>C. ulcerans</i> (99.7%)	<i>C. ulcerans</i>	<i>C. ulcerans</i> (99.6%)	<i>C. ulcerans</i> (99.6%)