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Application of an Improved Typing Method and Determination of the Antibiotic Susceptibility of Corynebacterium Strains

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Abstract: *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* are recognized as a veterinary pathogen and causing considerable economic impact due to the inefficacy of antimicrobial therapy. Diphtheria toxin can cause myocardial and neurological damage and diphtheria toxin gene detection is considered as a suitable procedure for determining the toxigenicity of corynebacterial isolates. Because there are few studies examining Corynebacterium in duck, researchers evaluated the incidence, toxigenicity and antibiotic susceptibility of Corynebacterium strains from moribund ducks in South Korea. In addition, researchers performed Direct Genome Restriction Enzyme Analysis (DGREA) for the subtyping of Corynebacterium strains. A total of 15 *C. pseudotuberculosis* strains and 2 *C. ulcerans* strains were identified using the Vitek System®2, PCR amplification and *16S rRNA* gene sequencing. All of the strains used in this study possessed the diphtheria toxin gene. Additionally, the results of antibiotic susceptibility testing showed that 15 of the 17 strains exhibited resistance to multiple antibiotics. In this study, DGREA yielded results that were dependent on the isolation area which could be useful for tracing the source of infection and indicate the potential application of this technique in disease prevention and disinfection.

Key words: *Corynebacterium pseudotuberculosis*, *corynebacterium ulcerans*, diphtheria toxin, ducks, Direct Genome Restriction Enzyme Analysis (DGREA), multiple antibiotic resistance

INTRODUCTION

Corynebacterium pseudotuberculosis is a Gram-positive bacterium associated with the development of abscesses in a variety of mammalian hosts causing ulcerative lymphangitis in horses and caseous lymphadenitis in small ruminants (Komala *et al*., 2008; Dorella *et al*., 2006). This bacterium is distributed globally and is problematic due to the inefficacy of antimicrobial therapy, additionally, it causes considerable economic impact (Piontkowski and Shivvers, 1998; Komala *et al*., 2008; Dorella *et al*., 2006).

C. ulcerans is primarily recognized as a veterinary pathogen and domestic animals may be potential reservoirs (Dewinter *et al*., 2005; De Zoysa *et al*., 2005). In addition, there has been a marked increase in the number of human infections (Dewinter *et al*., 2005; De Zoysa *et al*., 2005). However, there are few studies examining Corynebacterium in duck, although some studies have been performed in turkey (Saif *et al*., 2008). It is difficult to differentiate *C. pseudotuberculosis* from *C. ulcerans* because of the high genomic similarity between the two bacteria (Pacheco *et al*., 2007).

Diphtheria toxin is absorbed into the circulation and can cause myocardial and neurological damage (Christie, 1987). Although, diphtheria is now rare in developed countries, it is endemic in many developing countries (Pallen *et al*., 1994). Diphtheria toxin gene detection is a suitable procedure for determining the toxigenicity of corynebacterial isolates (Pallen *et al*., 1994).

In this study, researchers evaluated the incidence of *C. pseudotuberculosis* and *C. ulcerans* in Korean duck from October 2012 to May 2013. To determine the toxigenicity of corynebacterial isolates, researchers performed a PCR assay targeting the diphtheria toxin gene. Additionally, researchers evaluated the susceptibility of the isolates to 17 commercial antibiotics. Lastly, researchers performed Direct Genome Restriction Enzyme Analysis (DGREA) for the subtyping of Corynebacterium strains.

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MATERIALS AND METHODS

Bacterial strains and culture conditions: A total of 17 Corynebacterium strains (15 *C. pseudotuberculosis* strains and 2 *C. ulcerans* strains) were isolated from moribund ducklings exhibiting clinical signs of listlessness, ataxia, tremors of the head and legs and coma. The ducklings were obtained from 17 farms of Gyeonggi Province (n = 4), Chungcheongbuk Province (n = 3), Chungcheongnam Province (n = 4), Jeollabuk Province (n = 3) and Jeollanam Province (n = 3) in South Korea from October 2012 to May 2013 (Fig. 1). Brain swabs were streaked onto tryptic soy agar (TSA; Difco, USA) and incubated for 24 h at 37°C. All strains were stored at -80°C in 10% glycerol until they were used in experiments.

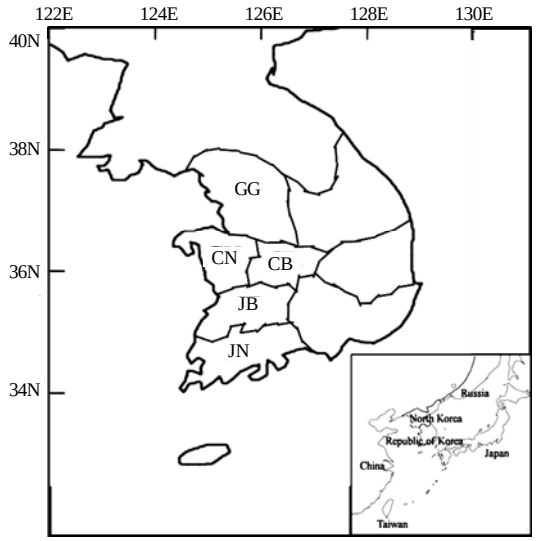

Fig. 1: Map showing the locations of the duck farms where Corynebacterium strains were isolated. GG: Gyeonggi Province, CB: Chungcheongbuk Province, CN: Chungcheongnam Province, JB: Jeollabuk Province, JN: Jeollanam Province

DNA isolation and molecular identification: Genomic DNA extraction was carried out via the small-scale preparation method of Sambrook *et al.* (1989). To isolate bacterial DNA, 1 mL of overnight bacterial culture in tryptic soy broth (TSB; Difco, USA) was collected via centrifugation. The pellet was washed with PBS and DNA was extracted using the Dneasy Tissue kit (Qiagen, Valencia, CA). The identities of all strains were analyzed via a multiplex PCR (mPCR) assay and *16S rRNA* gene sequencing. The oligonucleotide primers used in this study are listed in Table 1. Primers specific to the genus *Corynebacterium* (Cory52F and Cory1479R) were used (Tanner *et al*., 1999). The mPCR targeted three *C. pseudotuberculosis* genes: the *16S rRNA* gene which is the gene of choice for most microbial taxonomy studies (Cetinkaya *et al*., 2002; Khamis *et al*., 2005) *rpoB*, the RNA polymerase β-subunit gene currently used to study *Corynebacterium* genera (Khamis *et al*., 2004, 2005; Dorella *et al*., 2006) and *pld* which encodes the exotoxin PLD, a sphingomyelinase implicated in the virulence of *C. pseudotuberculosis* and *C. ulcerans* (McNamara *et al*., 1995). In this study, the primers PLD-F (which amplifies the *pld* genes of both *C. pseudotuberculosis* and *C. ulcerans*) and PLD-R2 (which amplifies the *pld* genes of *C. pseudotuberculosis* only) were used to exclude *C. ulcerans* strains. An mPCR that enables specific identification of *C. pseudotuberculosis* isolates were used in this study (Pacheco *et al*., 2007). Two primers (toxin-F and toxin-R) based on diphtheria toxin gene sequences were used to detect the production of diphtheria toxin in the isolates (Pallen *et al*., 1994).

The *16S rRNA* gene sequencing was performed by Macrogen Genomic Division (Seoul, Korea) and the sequenced genes of the bacterial strains acquired in this study were aligned with those of other bacteria of the same species and identified based on homology using BLAST.

Antibiotic susceptibility test: Antibiotic susceptibility testing was conducted via the Agar Disk Diffusion Method (Bauer *et al*., 1966) and the strains were determined to be resistant, intermediate or susceptible

Table 1: List of oligonucleotide primers used in this study

Target gene	Primers	Sequences (5'-3')	PCR products (bp)	References
Corynebacterial	Cory52F	GAACGCTGSCGGCGTGCTTAAC	1427	Murphy *et al.* (2005)
16S rRNA sequence[a]	Cory1479R	TTGTTACRRCTTCGTCCCAATCGCC		
16S rRNA gene[b]	16S-F	ACCGCACTTTAGTGTGTGTG	816	Jun *et al.* (2012)
	16S-R	TCTCTACGCCGATCTTGTAT		
rpoBI	C2700F	CGTATGAACATCGGCCAGGT	446	Jun *et al.* (2012)
	C3130R	TCCATTTCGCCGAAGCGCTG		
pld	PLD-F	ATAAGCGTAAGCAGGGAGCA	203	Jun *et al.* (2012)
	PLD-R2	ATCAGCGGTGATTGTCTTCCAGG		
Diphtheria toxin	toxin-F	ATCCACTTTTAGTGCGAGAACCTTCGTCA	248	Khamis *et al.* (2004)
	toxin-R	GAAAACTTTTCTTCGTACCACGGGACTAA		

[a]16S rRNA sequences specific to the genus *Corynebacterium*; [b]*16S rRNA* gene sequences of *C. pseudotuberculosis* and *C. ulcerans*

based on breakpoints in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). Commercially available antibiotic disks (Oxoid, England) were used in the assays. The antimicrobial classes utilized in the panel screens included aminoglycosides (gentamicin (CN), 10 μg), ansamycins (Rifampin (RD), 5 μg), carbapenems (Imipenem (IPM), 10 μg and Meropenem (MEM), 10 μg), cephems (cefepime (FEP), 30 μg; Cefotaxime (CTX), 30 μg and Ceftriaxone (CRO), 30 μg), folate pathway inhibitors (trimethoprim-Sulfamethoxazole (SXT), 1.25/23.75 μg), glycopeptides (Vancomycin (VA), 30 μg), lincosamides (clindamycin (DA), 2 μg), macrolides (Erythromycin (E), 15 μg), oxazolidinones (Linezolid (LZD), 30 μg), penicillins (Penicillin (P), 10 U), quinolones (Ciprofloxacin (CIP), 5 μg), streptogramins (Quinupristin-Dalfopristin (QD), 15 μg) and tetracyclines (Doxycycline (DO), 30 μg and Tetracycline (TE), 30 μg). All assays were performed using Muller-Hinton agar (Difco, USA) and the plates were incubated for 24 h at 37°C. The *Escherichia coli* strain ATCC 25922 was employed as a quality control.

Direct Genome Restriction Enzyme Analysis (DGREA): DGREA was performed as described previously by Fuenzalida *et al*. (2006) with modifications. Aliquots (10 μL) of DNA from each strain were digested with 5 U of the NdeI restriction enzyme (New England Biolabs, USA) according to the manufacturer's instructions and incubated at 37°C for 1 h. Each digestion product was resolved by electrophoresis on 7.5% polyacrylamide gels. The gels were run for 3 h at 100 V and stained using the PlusOne™ Silver Staining kit (GE Healthcare, USA).

Dendrogram and discrimination index: The genetic relationships among the isolates were assessed using Bionumerics Software (Applied Maths, Sint-Martens-Latem, Belgium) and the clusters were determined using the Unweighted Pair Group Method, Arithmetic mean (UPGMA) algorithm. The Hunter-Gaston discrimination index was calculated as described previously (Fuenzalida *et al*., 2006).

RESULTS

Isolation and identification of corynebacterium strains: A total of 17 Corynebacterium strains (15 *C. pseudotuberculosis* strains and 2 *C. ulcerans* strains) that were collected from moribund ducklings at 17 different farms in Korea were presumptively identified as *Corynebacterium* sp. using the Vitek System®2. PCR amplification and *16S rRNA* gene sequencing were performed using the 17 strains and the presence of the *rpoB*, *pld* and diphtheria toxin genes was determined, the results of these molecular analyses are summarized in Table 2. An analysis of *16S rRNA* gene sequences identified 17 strains as *Corynebacterium* sp., due to their high level of identity (100%, 8 strains; 99%, 9 strains).

Of the 17 strains, 15 were identified as *C. pseudotuberculosis* via PCR amplification of the *pld* gene of *C. pseudotuberculosis*. The other 2 strains were pld negative and were identified as *C. ulcerans*. In addition, all strains (15 *C. pseudotuberculosis* strains and 2 *C. ulcerans* strains) examined in this study possessed the diphtheria toxin gene.

Table 2: Corynebacterium strains from ducks with tremor used in this study

Strains	Isolation date (year)	Isolation area (province)	Vitek System®2 Probability (%)	PCR Cory	PCR 16S	PCR rpoB	PCR pld	PCR Toxin	16S rRNA sequence Sequence similarity (Accession No.)
C. pseudotuberculosis									
SNUCp-1	Oct. (2012)	Chungcheongnam	99	+[a]	+	+	+	+	99% (JF769750.1)
SNUCp-2	Oct. (2012)	Gyeonggi	99	+	+	+	+	+	99% (JF769750.1)
S NUCp-3	Oct. (2012)	Chungcheongnam	99	+	+	+	+	+	100% (HE983830.1)
SNUCp-4	Nov. (2012)	Gyeonggi	99	+	+	+	+	+	100% (JQ975896.1)
SNUCp-5	Nov. (2012)	Chungcheongnam	99	+	+	+	+	+	99% (JN834378.1)
SNUCp-6	Nov. (2012)	Jeollanam	98	+	+	+	+	+	100% (HE983829.1)
SNUCp-7	Feb. (2013)	Jeollabuk	99	+	+	+	+	+	99% (JF893647.1)
SNUCp-8	Feb. (2013)	Chungcheongnam	98	+	+	+	+	+	99% (JQ975896.1)
SNUCp-9	Feb. (2013)	Jeollanam	99	+	+	+	+	+	100% (KC311759.1)
SNUCp-10	Mar. (2013)	Jeollabuk	99	+	+	+	+	+	100% (JF460987.1)
SNUCp-11	Apr. (2013)	Jeollabuk	98	+	+	+	+	+	100% (JF769750.1)
SNUCp-12	Apr. (2013)	Jeollanam	99	+	+	+	+	+	99% (HQ183920.1)
SNUCp-13	Apr. (2013)	Chungcheongbuk	99	+	+	+	+	+	100% (JN584721.1)
SNUCp-14	May (2013)	Chungcheongbuk	99	+	+	+	+	+	99% (EU029498.1)
SNUCp-15	May (2013)	Chungcheongbuk	99	+	+	+	+	+	99% (EU438939.1)
C. ulcerans									
SNUCu-1	Oct. (2012)	Gyeonggi	99	+	+	+	-[b]	+	99% (NR074467.1)
SNUCu-2	Nov. (2012)	Gyeonggi	99	+	+	+	-	+	100% (NR074467.1)

[a]A positive reaction or the presence of a PCR product; [b]A negative or no PCR product

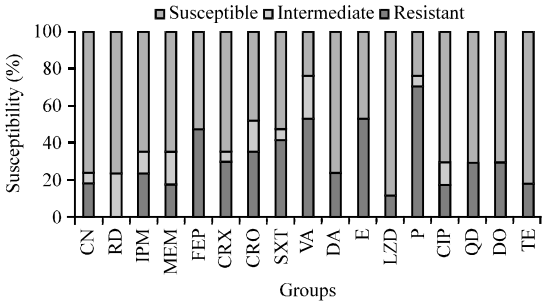

Fig. 2: An antibiotic susceptibility test of the isolated strains was performed using the disk diffusion method. The antibiotic resistance of the strains to 13 antimicrobial groups (17 commercial antibiotics) was explored. These groups included aminoglycosides (gentamicin (CN)), ansamycins (Rifampin (RD)), carbapenems (Imipenem (IPM) and Meropenem (MEM)), cephems (cefepime (FEP), Cefotaxime (CTX) and Ceftriaxone (CRO)), folate pathway inhibitors (trimethoprim-Sulfamethoxazole (SXT)), glycopeptides (Vancomycin (VA)), lincosamides (clindamycin (DA)), macrolides (Erythromycin (E)), oxazolidinones (Linezolid (LZD)), penicillins (Penicillin (P)), quinolones (Ciprofloxacin (CIP)), streptogramins (Quinupristin-Dalfopristin (QD)), tetracyclines (Doxycycline (DO) and Tetracycline (TE)). The antibiotic sensitivity of the isolated strains was determined by zone diameter interpretive standards (CLSI, 2006) and the percentages of isolates exhibiting Sensitive (S), Intermediate (I) and Resistant (R) phenotypes in the presence of various antibiotics are indicated

Antibiotic susceptibility test: The antibiotic resistance of the strains to 17 commercial antibiotics is illustrated in Fig. 2. Among the antibiotics tested in this study, resistance to penicillin (12 strains, 70.6%), erythromycin (9 strains, 52.9%) and vancomycin (9 strains, 52.9%) was most frequently observed. Of the 17 Corynebacterium strains, 15 exhibited multiple resistance (resistance to two or more antibiotics) whereas 2 strains (SNUCp-6 and SNUCu-2) did not. Significantly, a total of 15 *C. pseudotuberculosis* strains exhibited resistance to >5.8 antibiotics on average. No strain was found to be resistant to rifampin which was the most effective antibiotic agent.

DGREA typing: The *Corynebacterium* sp., groups were differentiated by DGREA of the total extracted bacterial DNA using the protocol described by Fuenzalida *et al.* (2006). In DGREA, 12-49 DNA fragments with sizes

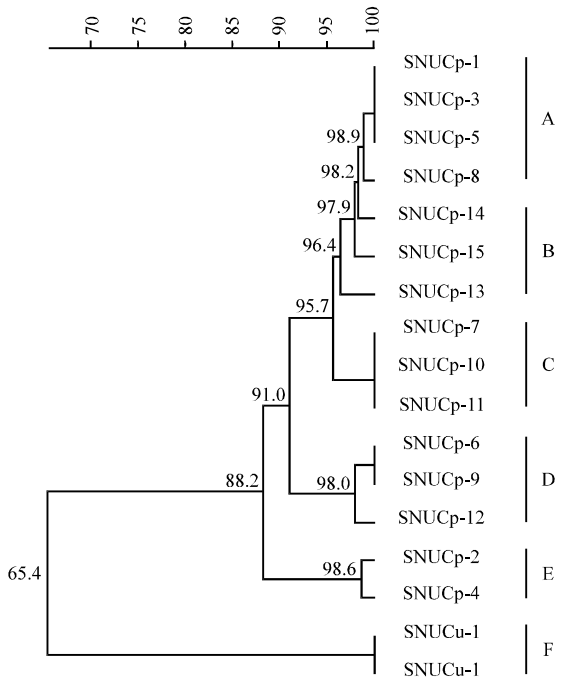

Fig. 3: Dendrogram illustrating the clusters of the patterns of the Corynebacterium strains with Direct Genome Restriction Enzyme Analysis (DGREA). The similarity units are shown

ranging from 300-10,200 bp were recognizable in the Corynebacterium strains. However, two *C. ulcerans* strains (group F) showed different digestion patterns from those of the *C. pseudotuberculosis* strains. The relationships among Corynebacterium strains were evaluated via cluster analysis of the patterns generated by DGREA (Fig. 3). The *C. pseudotuberculosis* strains were clustered into five groups (Fig. 3). Group A which is the predominant group (23.5% of the total strains), contains isolates from Chungcheongnam Province, groups B (17.6%), C (17.6%), D (17.6%) and E (11.8%) contain isolates from the Chungcheongbuk, Jeollabuk, Jeollanam and Gyeonggi Provinces, respectively. According to the results of DGREA analysis, compared with interspecies strains, all the *C. pseudotuberculosis* strains were more closely related and yielded a high discrimination index (0.875); additionally, they differed significantly from the *C. ulcerans* strains with a dissimilarity value of 34.6 (Fig. 3).

DISCUSSION

A total of 17 Corynebacterium strains were collected from 17 different duck farms covering 5 provinces of Korea (Gyeonggi, Chungcheongbuk, Chungcheongnam,

Jeollabuk and Jeollanam) to avoid overlapping analysis of a single strain. Researchers selected one representative strain from the moribund duck exhibiting the most severe clinical signs from each farm to analyze the regional differences and relationships among bacterial isolates. Because Corynebacterium is difficult to culture, it is often not identified in routine cultures which is recognized as a diagnostic problem (Domingue and Hellstrom, 1998; Tanner *et al*., 1999). The Vitek System®2 could not successfully identify all the strains in this study because the biochemical properties of *Corynebacterium* sp. are similar (Dewinter *et al*., 2005; Pacheco *et al*., 2007). Although, *16S rRNA* gene sequencing was useful for estimating the prevalence of Corynebacterium, it presented some limitations: first, it was dependent on bacterial culture and second, it was not specific enough to distinguish *C. pseudotuberculosis* from *C. ulcerans* (Cetinkaya *et al*., 2002; Pacheco *et al*., 2007). Cetinkaya *et al*. (2002) previously reported the high genomic similarity between *C. pseudotuberculosis* and *C. ulcerans*, revealing 99.7% similarity between their *16S rRNA* genes and 93.6% similarity between their *rpoB* genes (Khamis *et al*., 2004).

Avians are often suggested to be potential zoonotic reservoirs (Murphy *et al*., 2005). Researchers have reported that many human pathogenic organisms are carried by avians including ducks (Broman *et al*., 2002; Murphy *et al*., 2005). Although, *C. pseudotuberculosis* and *C. ulcerans* are capable of causing disease in humans and other animals these bacteria are poorly documented and the current understanding of their pathogenesis is incomplete (Murphy *et al*., 2005). Tanner *et al*. (1999) previously studied the prevalence of Corynebacterium in prostatitis patients and reported that *Corynebacterium* sp. were the most conspicuous organisms associated with prostatitis. Although, *C. ulcerans* is primarily recognized as a veterinary pathogen there has been a marked increase in the number of human infections (Dewinter *et al*., 2005; De Zoysa *et al*., 2005; Pacheco *et al*., 2007). All strains used in this study contained the diphtheria toxin gene, indicating the potential for these strains to cause zoonotic disease. Indeed, previous studies have noted that *C. pseudotuberculosis* and *C. ulcerans* eventually produce diphtheria toxin (Dewinter *et al*., 2005).

Antimicrobial resistance of food animals is a serious public health problem because antimicrobial resistant bacteria may be disseminated to humans via food processing chains. In the study, vancomycin-resistant strains (9 strains, 52.9%) were frequently observed. In contrast, previous reports indicated that all Corynebacterium strains were susceptible to vancomycin (Santamaria *et al*., 1985; Soriano *et al*., 1995). Significantly, Soriano *et al*. (1995) noted that vancomycin was the most effective antibiotic agent against *Corynebacterium* species. Potential resistance to vancomycin is an important point to consider because vancomycin has been frequently recommended as an empiric therapy for serious Gram-positive infections (Soriano *et al*., 1995). Although, macrolides (e.g., erythromycin) were considered as good therapeutic agents (Santamaria *et al*., 1985; Soriano *et al*., 1995), 9 (52.9%) of the 17 Corynebacterium strains proved resistant to erythromycin. Based on the results of the study, it is recommended that rifampin be used to treat corynebacterial infections as previously reported by other researchers (Santamaria *et al*., 1985; Soriano *et al*., 1995). Rifampin could be the drug of choice for the treatment of urinary tract infections including prostatitis because the concentration of this drug in urine was found to be maintained at a high level (Santamaria *et al*., 1985).

In this study, DNA sequencing-based typing methods such as single-locus sequence typing and multi-locus sequence typing were rejected due to the characteristics of *Corynebacterium* sp., including the wide sequence diversity of Corynebacterium strains and the high genomic similarity between *C. pseudotuberculosis* and *C. ulcerans*. DGREA is relatively rapid compared with Pulsed-Field Gel Electrophoresis (PFGE) which is very labor-intensive and time-consuming (Jun *et al*., 2012). Additionally, DGREA gives highly reproducible results and can be easily implemented with equipment available in any modern microbiology laboratory (Fuenzalida *et al*., 2006). The DGREA Method described herein proved to be a suitable method for the typing of *C. pseudotuberculosis* and *C. ulcerans*. This method differentiated *C. pseudotuberculosis* from *C. ulcerans* and could also differentiate intraspecific strains as reported previously by Fuenzalida *et al*. (2006) who studied *Vibrio parahaemolyticus*. Clustering based on DGREA did not coincide with patterns of antibiotic susceptibility, however, clustering corresponded exactly with the isolation area (province).

Although, it is thought that *C. pseudotuberculosis* and *C. ulcerans* do not to have the same potential to cause epidemic diphtheria as *C. diphtheria* (Pallen *et al*., 1994) future continuous monitoring of the prevalence and toxigenicity of these bacteria is recommended given that non-toxigenic Corynebacterium isolates carrying the diphtheria toxin gene are quite rare globally (Pallen *et al*., 1994). Because most of the available data regarding antibiotic susceptibility are derived from scattered case reports, studies on a particular organism or very old reports (Soriano *et al*., 1995) the results could be more useful when prescribing antibiotics in cases of Corynebacterium infection in humans or animals.

CONCLUSION

To the best of the knowledge this is the first description of the use of DGREA for subtyping *Corynebacterium* sp. Additionally in this study, DGREA yielded results that were dependent on isolation area which could be useful for tracing the source of infection and indicate the potential application of this technique in disease prevention and disinfection.

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