Prevalence of *Corynebacterium ulcerans* in dogs in Osaka, Japan

Chihiro Katsukawa,¹ Takako Komiya,² Hiroaki Yamagishi,³ Atsushi Ishii,³ Shunji Nishino,³ Shinya Nagahama,⁴ Masaaki Iwaki,² Akihiko Yamamoto² and Motohide Takahashi²

Correspondence

Chihiro Katsukawa
katukawa@iph.pref.osaka.jp
Masaaki Iwaki
miwaki@nih.go.jp

¹Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka, Japan

²Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan

³Osaka Prefectural Dog Management Office, Osaka, Japan

⁴Animal Protection and Livestock Division, Department of Environment, Agriculture, Forestry and Fisheries, Osaka Prefectural Government, Osaka, Japan

Diphtheria-like human illness caused by *Corynebacterium ulcerans* is an emerging threat in developed countries, with incidence sometimes higher than that of diphtheria caused by *Corynebacterium diphtheriae*. Companion animals are considered a potential source of human infections. In order to determine the prevalence of *C. ulcerans* among dogs, we performed a screening for the bacterium in 583 dogs in the custody of the Osaka Prefectural government. Forty-four dogs (7.5%) were positive for the bacterium, although they did not show any clinical symptoms. All bacterial isolates showed resistance or decreased sensitivity to clindamycin, and some showed decreased sensitivity to levofloxacin. Comparative analysis of isolates using PFGE, toxin gene typing and antibiotic sensitivities suggests that transmission between asymptomatic dogs might have occurred.

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INTRODUCTION

Some strains of *Corynebacterium ulcerans* produce diphtheria toxin and may cause human infection with diphtheria-like symptoms. In Japan, eight cases of infection by toxigenic *C. ulcerans* in humans were reported between 2001 and 2011 (Asakura *et al.*, 2006; Hagiwara *et al.*, 2006; Hatanaka *et al.*, 2003, 2011; Komiya *et al.*, 2010; Noguchi *et al.*, 2009; Nureki *et al.*, 2007; Yoshimura *et al.*, 2010). *C. ulcerans* causes purulent inflammatory diseases such as mastitis, lymphadenitis, dermatitis and respiratory infections in various domestic and wild animals. Human infections are caused by ingestion of untreated milk (Bostock *et al.*, 1984) or by close contact with a variety of animals (De Zoysa *et al.*, 2005; Hatanaka *et al.*, 2011; Noguchi *et al.*, 2009; Schuëgger *et al.*, 2009; Tiwari *et al.*, 2008; Yoshimura *et al.*, 2010). Dogs are of pronounced importance because they are sometimes linked to serious human cases (Hogg *et al.*, 2009; Lartigue *et al.*, 2005).

Abbreviations: CLDM, clindamycin; CLSI, Clinical and Laboratory Standards Institute; LVFX, levofloxacin.

The GenBank/EMBL/DBJ accession numbers for the *tox* sequences of *C. ulcerans* Dog0803, Dog0804 and Dog0811 and *C. diphtheriae* ATCC 11049, ATCC 11051, RIMD 0343044 and CD1994-1 are AB602353, AB602354, AB602355, AB602356, AB602357, AB602358 and AB602359, respectively.

Supplementary tables are available with the online version of this paper.

To determine whether domestic dogs serve as a reservoir for human *C. ulcerans* infection, we carried out a series of initial screenings for *C. ulcerans* in 65 healthy dogs who were, for various reasons, in the custody of the Osaka Prefectural Government from December 2006 to September 2007. Toxigenic *C. ulcerans* was isolated from one dog (Katsukawa *et al.*, 2009). *C. ulcerans* was not found in any of the 218 family dogs living in the area where the infected dog originated (Takahashi, 2009).

In this study, we report the results of our continuation of the screening for *C. ulcerans* in the dogs in custody. In order to compensate for any seasonal effects on the screening, the screening period was extended to cover 13 months from November 2007 to December 2008. Upon examination of 583 dogs, 45 *C. ulcerans* isolates were obtained from 44 dogs. The characteristics of these isolates are described here.

METHODS

Dogs and sample collection. Between 27 November 2007 and 26 December 2008, 944 adult dogs were under the care of the Osaka Prefectural Government. Due to a tight schedule for euthanizing the dogs, throat swabs were collected from only 583 of the 944 dogs. The test population included 363 male and 220 female (401 mongrels and 182 purebreds) dogs. These dogs were either abandoned or had escaped from their guardians. Throughout the screening period,

sample collection was usually carried out twice a week, on Tuesdays and Fridays. Throat swabs were collected immediately after euthanasia and stored in preservation medium (SEEDSWAB γ 3 'Eiken'; Eiken Chemical) at 4 °C.

Bacterial isolation and identification. Bacterial strains used in this study are listed in Table 1. For the isolation of bacteria, culturing was started on the day of sample collection by inoculating the swabs on sheep blood agar and selective medium (charcoal–tellurite blood agar), and incubating them at 35 °C (Katsukawa *et al.*, 2009). Charcoal–tellurite blood agar contains heart infusion agar, 0.03 % (w/v) potassium tellurite, 10 % (v/v) sheep blood and 0.05 % (w/v) activated charcoal (Katsukawa *et al.*, 2009). Colonies appearing after 18–24 h on sheep blood agar and after 24, 30 and 48 h on charcoal–tellurite blood agar were transferred to dextrose–sucrose–starch agar medium (Jacherts, 1956) to investigate glucose and sucrose fermentation. Isolates positive for glucose but negative for sucrose fermentation were then characterized by Gram staining and by the catalase and urease tests. Thus, all Gram-positive organisms that tested positive for catalase and urease production were suspected to be *C. ulcerans* and further analysed using an API Coryne (bioMérieux) kit, followed by the determination of partial RNA polymerase β -subunit (*rpoB*) sequences (Khamis *et al.*, 2004).

Toxigenicity testing and nucleotide sequencing of the *tox* gene. Toxigenicity testing was performed by modified Elek test (Engler *et al.*, 1997; Katsukawa *et al.*, 2009; Reinhardt *et al.*, 1998), Vero cell cytotoxicity and neutralization tests (Katsukawa *et al.*, 2009;

Miyamura *et al.*, 1974) and PCR for the diphtheria toxin (*tox*) gene. Primers used for PCR are listed in Supplementary Table S1 in JMM Online. Primers Tox 1 and Tox 2 (Nakao & Popovic, 1997), corresponding to the A subunit of the toxin, were first used for detection of the *tox* gene. In isolates negative for the primer pair, further PCR analyses using primers Dipht 6F and Dipht 6R (Nakao *et al.*, 1996) for the B subunit and toxFw and toxRv for the entire *tox* gene (Seto *et al.*, 2008) were carried out to confirm the absence of the gene. The nucleotide sequence of the *tox* gene was determined by amplifying the entire gene fragment with primers toxFw and toxRv (Seto *et al.*, 2008) (Supplementary Table S1) and then by sequencing the fragment with primers placed at appropriate intervals. A phylogenetic tree of *tox* nucleotide sequences was constructed using the neighbour-joining method (Saitou & Nei, 1987).

PFGE. PFGE was performed on all 45 isolates as well as on the *C. ulcerans* strains previously isolated from dogs and humans in Japan. PFGE of *Sfi*I-digested genomic bacterial DNA (De Zoysa *et al.*, 1995) was performed using 1.5 % (w/v) agarose gels and 0.5 $\times$ Tris/borate EDTA buffer at 14 °C, 6 V cm⁻¹, 5–20 s pulse for 18 h, followed by 1–5 s pulse for 14 h, using a CHEF-DR II system (Bio-Rad). PFGE patterns were analysed with the Diversity Database software by the UPGMA algorithm.

Antibiotic susceptibility. Antibiotic susceptibility tests were performed by the broth microdilution method using a Dry Plate DP24 (Eiken Chemical). We tested the following antibiotics: benzylpenicillin, ampicillin, cefazolin, cefotiam, cefotaxime, cefaclor, cefditoren, flomoxef, imipenem, meropenem, erythromycin, clindamycin (CLDM), minocycline, vancomycin, levofloxacin (LVFX) and sulfamethoxazole–trimethoprim. Sensitivities were assessed according to the Clinical and Laboratory Standards Institute's (CLSI) standard (M45-A) for *Corynebacterium* species. To assign sensitivities to the eight drugs for which CLSI standard values are not available, standards for similar drugs were considered (sensitivity to ampicillin tested using benzylpenicillin sensitivity standard; cefazolin, cefotiam, cefaclor, cefditoren and flomoxef tested using cefotaxime standard; minocycline tested using tetracycline standard; and LVFX tested using ciprofloxacin sensitivity standard).

Table 1. Bacterial strains and isolates used in this study

Strains used for comparison studies are listed here.

Isolate/strain	Source	Reference
<i>Corynebacterium ulcerans</i>		
0102	Human	Hatanaka <i>et al.</i> (2003); Komiya <i>et al.</i> (2010)
0211	Human	Komiya <i>et al.</i> (2010)
0509	Human	Asakura <i>et al.</i> (2006)
0510	Human	Nureki <i>et al.</i> (2007)
0607	Human	Hagiwara <i>et al.</i> (2006)
0902	Human	Noguchi <i>et al.</i> (2009)
Dog0708	Dog	Katsukawa <i>et al.</i> (2009)
Dog0803	Dog	This study
Dog0804	Dog	This study
Dog0807	Dog	This study
Dog0809-1	Dog	This study
Dog0809-2	Dog	This study
Dog0809-3	Dog	This study
Dog0811	Dog	This study
<i>Corynebacterium diphtheriae</i>		
ATCC 11049	Human	ATCC
ATCC 11051	Human	ATCC
RIMD 0343044	Human	RIMD*
CD1994-1	Human	Thailand (this study)

*Culture Collection of the Research Institute for Microbial Diseases, Osaka University, Japan.

RESULTS

Isolation of *C. ulcerans* and toxigenicity

Fig. 1 displays the number of isolates obtained over the course of the study. Supplementary Table S2 also summarizes the study population with respect to gender and seasonality of *C. ulcerans* isolation. A more comprehensive summary of the *C. ulcerans* isolates is shown in Table 2.

The first isolate was detected in March 2008, followed by the isolation of indistinguishable isolates from three other dogs that had the same guardian. These four isolates were classified as group 1. Screening continued intermittently, with 27 *C. ulcerans*-positive dogs identified between April and the beginning of June (group 2), six in July (group 3) and none until September. Seven isolates were obtained from six dogs in September and October (group 4, subdivided into three subgroups, 4-1 to 4-3, by toxigenicity and PFGE patterns) and one isolate from one dog in November (group 5). In summary, 45 *C. ulcerans* isolates (42 toxigenic and three non-toxigenic) were obtained from 44 dogs. From one of these dogs, toxigenic and

Fig. 1. Chronological representation of *C. ulcerans* isolation. Samples were collected as described in the text. Each sampling period is expressed as a small cell between the horizontal axis and month indications. Bars represent the number of isolates obtained during each sampling period.

non-toxicogenic isolates were isolated simultaneously (Table 2, nos 39 and 40). For each of the other dogs, one isolate was obtained per dog (41 toxicogenic and two non-toxicogenic). None of the 44 dogs from which *C. ulcerans* was isolated were apparently symptomatic: nasal discharge, pharyngitis and dermatitis were not observed. There was no correlation between detection rates and the sex of the animals.

Each of the 45 isolates were obtained from the primary culture growing on charcoal–tellurite blood agar plates. Isolation from primary cultures on blood agar was not successful: only three isolates were obtained, mainly due to masking by the growth of other bacterial species present on the swab. The three isolates were later proven to be identical to the isolates obtained from the charcoal–tellurite blood agar plates of the same specimens.

All strains tested positive for catalase and urease production and glucose fermentation and negative for sucrose fermentation. They shared the same API code (0111326, % id 99.7, $T=1.0$). Forty-two isolates were toxicogenic, as demonstrated by positive results of PCR for the *tox* gene (A subunit), modified Elek test, and Vero cell cytotoxicity and neutralization tests. Three isolates were negative for all of these tests and were thus considered to be non-toxicogenic. Detailed characteristics of these isolates are summarized in Table 2.

PFGE analysis

All 45 isolates and the previous dog and human isolates were compared using PFGE. The isolates were classified into four types (A2, B, C, D) by PFGE (Table 2 and Fig. 2). PFGE classifications of isolates were as follows: PFGE type C included group 1 isolates (four toxicogenic strains isolated in March); type A2 included groups 2, 3, 4-1 and 4-2 (37 toxicogenic strains isolated from April to October and two of

three non-toxicogenic strains isolated in September); type B included group 4-3 (one non-toxicogenic strain isolated in September); and type D included group 5 (one toxicogenic strain isolated in November). As shown in Fig. 2, subtype A2, together with two other subtypes classified according to slight differences in PFGE patterns, composed type A. Previously isolated human strains have been classified with respect to PFGE type as follows: PFGE type A1, human isolates 0102 and 0210 (Komiya *et al.*, 2010) that were closely related to type A2, including 0509 (Asakura *et al.*, 2006) and 0902 (Noguchi *et al.*, 2009), and type A3 including 0607 (Hagiwara *et al.*, 2006). Another human isolate, 0510 (Nureki *et al.*, 2007), was classified in a comparatively distant type D.

Analysis of the diphtheria toxin (*tox*) gene

The complete *tox* gene sequence (1683 bp) was determined for 42 toxicogenic isolates for which toxigenicity was confirmed. Their sequences were divided into three types as shown in Table 2 and Fig. 3. The first type (designated tox0803; GenBank accession no. AB602353) included four isolates of group 1 including Dog0803; the second type (tox0804; GenBank accession no. AB602354) included 37 toxicogenic isolates of groups 2, 3 and 4-1, obtained from April through October, including Dog0804; and the last type (tox0811; GenBank accession no. AB602355) included only one isolate, Dog0811 (group 5), obtained in November. These sequences were also compared to the previously published *tox* gene sequences of *C. ulcerans* human strains 0102, 0510, A6361, A2911, X959 and KL126 (Komiya *et al.*, 2010; Schuëgger *et al.*, 2009; Seto *et al.*, 2008; Sing *et al.*, 2003, 2005; Wellinghausen *et al.*, 2002) (Table 3, Fig. 3). More than 98 % similarity was shown between any two of the *C. ulcerans* isolates and strains. However, the sequences showed reduced similarity (~95 %) to the reported *tox* gene

Table 2. Characteristics of the isolates

No.	Group	Designation as the isolate representing the group	Date of isolation*	Sex†	Isolation on‡:		API Coryne code	tox PCR	Elek	Vero cell cytotoxicity	PFGE type	tox gene type	Drug susceptibility§
					BA	C							
1	1	Dog0803	3/11	F	—	+	0111326	+	+	+	C	tox0803	CLDM: R
2	1		3/11	M	—	+	0111326	+	+	+	C	tox0803	CLDM: R
3	1		3/14	F	—	+	0111326	+	+	+	C	tox0803	CLDM: R
4	1		3/14	F	—	+	0111326	+	+	+	C	tox0803	CLDM: R
5	2	Dog0804	4/4	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
6	2		4/11	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
7	2		4/15	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
8	2		4/15	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
9	2		4/15	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
10	2		4/15	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
11	2		4/22	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
12	2		4/22	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
13	2		4/22	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
14	2		5/2	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
15	2		5/2	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
16	2		5/2	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
17	2		5/9	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
18	2		5/9	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
19	2		5/9	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
20	2		5/13	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
21	2		5/13	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
22	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
23	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
24	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
25	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
26	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I
27	2		5/13	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
28	2		5/20	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
29	2		6/3	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: I
30	2		6/3	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: I
31	2		6/3	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: R
32	3	Dog0807	7/15	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
33	3		7/18	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
34	3		7/22	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
35	3		7/25	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
36	3		7/25	F	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
37	3		7/25	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I, LVFX: I
38	4-1	Dog0809-1	9/16	M	+	+	0111326	+	+	+	A2	tox0804	CLDM: I
39	4-1		9/26	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I
40	4-2	Dog0809-2	9/26	M	—	+	0111326	—	—	—	A2	—	CLDM: I
41	4-3	Dog0809-3	9/26	M	—	+	0111326	—	—	—	B	—	CLDM: I
42	4-2		9/26	M	—	+	0111326	—	—	—	A2	—	CLDM: I
43	4-1		9/26	M	+	+	0111326	+	+	+	A2	tox0804	CLDM: I
44	4-1		10/7	M	—	+	0111326	+	+	+	A2	tox0804	CLDM: I
45	5	Dog0811	11/25	M	+	+	0111326	+	+	+	D	tox0811	CLDM: R

*Month/day in 2008.

†M, Male; F, female.

‡BA, Blood agar; C, charcoal–tellurite blood agar.

§CLDM, Clindamycin; LVFX, levofloxacin; R, resistant; I, intermediate.

||Isolated from the same dog.

Fig. 2. Phylogenetic analysis of *C. ulcerans* isolates. *C. ulcerans* dog isolates obtained in this study (indicated in bold) as well as Japanese human isolates reported previously (Asakura *et al.*, 2006; Hagiwara *et al.*, 2006; Hatanaka *et al.*, 2003; Komiya *et al.*, 2010; Noguchi *et al.*, 2009; Nureki *et al.*, 2007) were analysed by PFGE as described in Methods. The phylogenetic tree was generated by the UPGMA method.

of the *C. diphtheriae* NCTC 13129 genome sequence strain. We also determined the complete *tox* gene sequences of *C. diphtheriae* culture collection strains ATCC 11049 (GenBank accession no. AB602356), ATCC 11051 (GenBank accession no. AB602357) and RIMD 0343044 (GenBank accession no. AB602358) and of a clinical isolate, CD1994-1 (GenBank accession no. AB602359). These *tox* sequences differed from that of the *C. diphtheriae* genome sequence but only by 0–2 bases. The deduced amino acid sequences of all five *C. diphtheriae* strains were identical.

Antibiotic sensitivity

None of the strains were resistant to benzylpenicillin, ampicillin, cefazolin, cefotiam, cefotaxime, cefaclor, cefditoren, flomoxef, imipenem, meropenem, erythromycin, minocycline, vancomycin or sulfamethoxazole–trimethoprim. The CLDM MIC was $\geq 2 \mu\text{g ml}^{-1}$ in all strains, judged as resistant or intermediate. In addition, the LVFX

MIC was $2 \mu\text{g ml}^{-1}$ in six isolates and $\leq 0.25 \mu\text{g ml}^{-1}$ in the remaining 39 isolates.

DISCUSSION

In this study, 42 toxigenic and three non-toxigenic *C. ulcerans* isolates were obtained from dogs in the custody of the Osaka Prefectural Government from November 2007 to December 2008. The isolates were divided into five groups and three subgroups according to the period of isolation, as shown in Table 2. Further analysis showed that these groupings correlated with PFGE types and *tox* gene sequence types.

Group 1 isolates were obtained from four (50%) of eight dogs with the same guardian, suggesting a group infection by *C. ulcerans* in an asymptomatic state. The guardian was looking after these dogs in a pasture in the riverbed. The source of infection was unclear; however, many types of

Fig. 3. Phylogenetic analysis based on nucleotide sequences of the *tox* genes. The *tox* gene sequences for the *C. ulcerans* isolates obtained in the present study were determined and compared to the previously published *tox* gene sequences of *C. ulcerans* strains isolated from humans and the *C. diphtheriae* strain NCTC 13129. A dendrogram was generated by the neighbour-joining method (Saitou & Nei, 1987).

Table 3. Nucleotide sequence (roman) and deduced amino acid (italic>) similarity (%) of *tox* genes

<i>tox</i> type or strain	<i>Corynebacterium ulcerans</i>						<i>C. diphtheriae</i>
	tox0811	A2911	tox0804	tox0803	KL126	X959	NCTC 13129
tox0811		98.9	99.1	98.9	98.8	98.6	94.8
A2911	98.5		99.5	99.3	99.1	98.6	94.8
tox0804	98.6	99.4		99.8	99.6	99.1	95.0
tox0803	98.5	99.1	99.7		99.8	99.3	94.8
KL126	98.5	99.0	99.6	99.9		99.5	94.7
X959	98.4	98.9	99.5	99.8	99.8		94.5
NCTC 13129	95.1	95.0	95.1	95.2	95.1	95.1	

animals inhabit the fields, and they possibly played a role in *C. ulcerans* transmission.

Group 2 organisms were isolated from 27 (28 %) of 98 dogs examined in 2 months from April 4 to June 3. These dogs came, not from a specific area, but from a wide range of locations in the Osaka Prefecture. Dogs residing in the Osaka Prefectural Dog Management Office were usually euthanized after being boarded for about 1 week. Because dogs at the Dog Management Office were not kept in isolation, they had many opportunities for physical contact. Therefore, *C. ulcerans* was possibly transmitted from one dog to another during the boarding period in the facility. This hypothesis is supported by our results indicating identical PFGE and *tox* gene sequence types for all isolates belonging to group 2. Furthermore, after group 2 dogs were cleared from the boarding facility after sampling on June 3, *C. ulcerans* was not detected in the 45 dogs tested during the subsequent sampling period.

Group 3 was composed of a cluster of isolates obtained subsequent to group 2. This group (six isolates) had the same PFGE type (A2) as group 2 and group 4-1 isolates; however, the LVFX MIC in group 3 isolates was higher than that for group 2 and group 4-1 isolates. We therefore concluded that group 3 isolates originated from different sources to those for the isolates in groups 2 and 4-1. As with group 2, each of the six dogs in group 3 was transported to the facility from geographically distant regions of Osaka Prefecture. Thus, these *C. ulcerans* isolates might have been transmitted from one dog to another in the facility, rather than acquired from dogs residing at their original locations.

The next cluster of isolates was designated group 4, isolated from six dogs in September and October. Although transmission is assumed to have occurred within the facility, four strains were toxigenic while the other three were not. Two distinct PFGE patterns for the three isolates (A2 for nos 40 and 42, and B for no. 41) characterized non-toxigenic strains, indicating their different origins. In contrast, isolate nos 39 and 40 (one toxigenic and the other non-toxigenic, respectively) were obtained from a single dog (Table 2). Apart from their toxigenicity, these isolates were indistinguishable, sharing the same API code, PFGE type and antibiotic resistance pattern. The *tox* gene

of *C. ulcerans* is known to be carried by bacteriophages (Seto *et al.*, 2008). Toxigenicity in these two isolates might be the result of *tox* gene acquisition through infection by and lysogenization of a bacteriophage. Alternatively, the non-toxigenic isolate might have been the result of loss of *tox*-bearing bacteriophage from the toxigenic organism. Further analysis will support this hypothesis if isogenicity between these isolates is confirmed.

Additionally, another isolate was obtained in November and was classified in group 5. This isolate exhibited an independent PFGE pattern from those of the other isolates (Table 2, Figs 1 and 2).

Although *C. ulcerans* can cause mouth ulcers (Lartigue *et al.*, 2005) and bronchopneumonia (Sykes *et al.*, 2010), all *C. ulcerans*-positive dogs were asymptomatic, regardless of the toxigenicity of the isolate. Our studies suggest that although weakly virulent, *C. ulcerans* is readily transmitted among dogs. The lack of *C. ulcerans* isolation in winter suggests that fewer contacts occurred between the animals during cold weather due to reduced activity.

In our previous study in 2009 (Katsukawa *et al.*, 2009), the rate of incidence (1/65) was considerably lower than the incidence (45/583) in the present study. The apparent discrepancy might be due to the difference in the sampling population: in the previous study, the population examined was supposed to be less than 10 % of the total number of dogs in the custody, and the results obtained there might not have reflected the actual carrier rate.

Among the 45 *C. ulcerans* isolates, 42 were toxigenic. The phylogenetic tree and sequence comparisons are shown in Fig. 3 and Table 3, respectively. All *tox* genes of *C. ulcerans* formed a monocluster distinct from that of the *tox* gene of *C. diphtheriae*, which is in agreement with the results of previous studies (Seto *et al.*, 2008; Sing *et al.*, 2003, 2005). It is thus unlikely that the *tox* genes detected in the *C. ulcerans* isolates obtained in this study could have been transmitted from *C. diphtheriae*. In Brazil, a non-toxigenic *C. ulcerans* strain has also been isolated from a dog kept in an animal shelter (Dias *et al.*, 2010). In this case, transmission between dogs was not observed.

C. diphtheriae-selective medium containing potassium tellurite has been routinely used for years (Efstratiou &

George, 1999), but its selective ability for other corynebacteria has not been fully evaluated. Using the broth (Mueller–Hinton broth) microdilution method, we found that the MIC of potassium tellurite for *C. ulcerans* and *C. diphtheriae* was 0.03 % and 0.125 %, respectively. The cell density of *C. ulcerans* at 0.03 % potassium tellurite was fourfold less than that of *C. diphtheriae* at 0.125 % (data not shown). Tinsdale agar medium, which is frequently used to selectively isolate *C. diphtheriae* (Tinsdale, 1947), has been assumed to support the growth of *C. ulcerans* as well. This medium contains approximately 0.03 % potassium tellurite; however, its concentration differs according to the manufacturer. Therefore, *C. ulcerans* strains could show relatively poor growth on Tinsdale agar. As reported previously, a medium was developed for the efficient culture of *C. ulcerans* (Katsukawa *et al.*, 2009). By adding activated charcoal and blood to Mueller–Hinton broth, the MIC for *C. ulcerans* increased to 0.125 %. We therefore used charcoal–tellurite blood agar, which contains activated charcoal (0.05 %), potassium tellurite (0.03 %) and sheep blood (10 %); the composition was based on heart infusion agar, which is used to detect *C. ulcerans*. This agar medium enabled us to isolate *C. ulcerans* from 44 of 583 dogs in the present study. All *C. ulcerans* isolates were obtained from the charcoal–tellurite blood agar, but only three from the blood agar. These data show that charcoal–tellurite blood agar is suitable for the selective isolation of *C. ulcerans* from samples that contained small numbers of *C. ulcerans* and many other normal flora bacteria from dogs' throats.

The strains isolated in this study were all sensitive to β -lactam or macrolide antibiotics, which are often used to treat diphtheria (Bonnet & Begg, 1999). In contrast, all strains were judged as intermediate resistant or resistant to CLDM, and six strains were intermediately resistant to LVFX. Although reports of erythromycin- and CLDM-resistant (Tiwari *et al.*, 2008), erythromycin-resistant (Schuhegger *et al.*, 2009; Tiwari *et al.*, 2008) and CLDM-intermediate resistant strains (Sykes *et al.*, 2010) have been published, resistance to quinolones has not been reported until now. A report of failure in curing the bacterium by enrofloxacin, another fluoroquinolone antibiotic, has been described (Sykes *et al.*, 2010). Since the CLSI does not publish a standard LVFX MIC for *Corynebacterium* species, six strains with an LVFX MIC of $2 \mu\text{g ml}^{-1}$ could not be classified as susceptible. The increased resistance of some *C. ulcerans* strains to LVFX indicates that care should be taken in administering quinolones to treat infections.

Corynebacterial infections in dogs are difficult to cure: amoxicillin (2 g daily for 15 days), enrofloxacin (5 mg kg^{-1}) and doxycycline (5.8 mg kg^{-1} orally every 12 h for 10 weeks) treatments have been reported to be unsuccessful (Hogg *et al.*, 2009; Lartigue *et al.*, 2005; Sykes *et al.*, 2010). Mechanisms to account for the ineffectiveness of these antibiotics remain to be determined. The drugs may be less effective due to metabolic factors in the host, an inability to access the bacteria, or inefficient transport into the

pathogen. Alternatively, the host may be repeatedly infected.

Since there would be great resistance to euthanizing companion animals to eliminate a bacterial reservoir, controlling infections by an antibiotic-independent way is of utmost importance. Development of an effective vaccine to prevent animals from infection may provide an alternative to antibiotic treatment. Existing diphtheria toxoid vaccines are considered to be effective in protecting humans from toxæmic diseases caused by *C. ulcerans* (De Zoysa *et al.*, 2005; Tiwari *et al.*, 2008). However, the toxoid vaccine is not considered to prevent animals and humans from infection because its nature is chemically or physically detoxified diphtheria toxin. Vaccines effective against infection, possibly by utilizing bacterial components essential for colonization, are thus desired, especially because non-toxigenic *C. ulcerans* can be converted to toxigenic by bacteriophages. Additional studies need to be conducted to identify candidate antigens for a suitable animal vaccine.

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