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# The detection of toxigenic *Corynebacterium ulcerans* from cats with nasal inflammation in Japan

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## SUMMARY

*Corynebacterium ulcerans* (toxigenic *C. ulcerans*) produces the diphtheria toxin, which causes pharyngeal and cutaneous diphtheria-like disease in people, and this bacterium is commonly detected in dogs and cats that are reared at home. It is considered dangerous when a carrier animal becomes the source of infection in people. To investigate the carrier situation of toxigenic *C. ulcerans* of cats bred in Japan, bacteria were isolated from 37 cats with a primary complaint of rhinitis in 16 veterinary hospitals in Osaka. Toxigenic *C. ulcerans* was detected in two of the cats. By drug sensitivity testing, the detected bacterium was sensitive to all investigated drugs, except clindamycin. It appears necessary to create awareness regarding toxigenic *C. ulcerans* infection in pet owners because this bacterium is believed to be the causative organism for rhinitis in cats.

**Key words**: Cat, Japan, Osaka, rhinitis, toxigenic *Corynebacterium ulcerans*.

## INTRODUCTION

*Corynebacterium ulcerans* is widely distributed in the environment, and it is considered a commensal bacterial species in domestic and wild animals. It is known that this bacterium can cause cutaneous inflammation including mastitis in dairy cows [1–3]. Recently, the microorganism has been increasingly recognized as an emerging zoonotic agent of diphtheria-like illness. In humans, diphtheria is an upper respiratory tract illness caused by *C. diphtheria*, and it is characterized by sore throat, low-grade fever, and the formation of a pseudomembrane on the tonsils, pharynx, and/or nasal cavity [4]. However, *C. ulcerans* has been detected in the pharynx of patients with diphtheria-like symptoms, and the isolates appear to produce diphtheria toxin (this type of *C. ulcerans* is referred as toxigenic *C. ulcerans*) [5–7]. The toxigenic *C. ulcerans* strains harbour lysogenic beta-corynephages bearing the *tox* gene, which encodes the diphtheria toxin, and are thus responsible for the systemic symptoms of diphtheria [8]. In recent epidemiological surveys, toxigenic *C. ulcerans* was isolated from companion animals such as dogs and cats, and livestock such as cows and sheep; thus, these animals were strongly implicated as sources of human infections [7, 9, 10].

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Table 1. *Information on cats with* Corynebacterium ulcerans *isolated and summary of clinical findings*

| No. | Breed | Sex | Age (years) | Feeding period (years) | Habitat | Clinical history | Virus (FIV/FeLV) |
|---|---|---|---|---|---|---|---|
| 7 | Mongrel | F | 3 | 3 | Free roaming | Chronic sneeze, purulent nasal discharge | Not done/– |
| 32 | Mongrel | M | 2 | 2 | Free roaming | Rhinitis, breathing difficulty | –/– |

FIV, Feline immunodeficiency virus, FeLV, feline leukaemia virus.

Twelve cases of diphtheria or diphtheria-like disease associated with toxigenic *C. ulcerans* have been reported in Japan since toxigenic *C. ulcerans* was first detected in 2001 [11–17]. In several cases, patients were confirmed as having had direct contact with dogs or cats with/without dermatitis or respiratory symptoms such as rhinitis [11, 17]. The genotypes of toxigenic *C. ulcerans* isolates from patients were identical to those of the stray cats that they had physical contact with [15]. More recently, toxigenic *C. ulcerans* was detected in domestic dogs and cats in Japan via molecular approaches [17, 18]. In Japan, people generally have close contact with these companion animals more frequently than livestock and share living environments with their pets. In veterinary hospitals, clinical symptoms of rhinitis are frequently identified in cats; however, infection with *C. ulcerans* in these pets, particularly infection by toxigenic *C. ulcerans*, remains unclear. In the present study, we investigated *C. ulcerans* infection in pet cats with clinical symptoms and characterized the isolates, including possession of the *tox* gene, to assess the potential risk to humans.

## MATERIALS AND METHODS

### Animals

In total, 37 cats that were brought to animal hospitals (16 hospitals) in Osaka, Japan between October 2009 and March 2010 were included in the study. The animals displayed chronic rhinitis (Table 1) and their nasal discharge or rhinal secretions were collected using a cotton swab (Seedswab no. 2, Eiken Chemical Co. Ltd, Japan) for examination.

### Isolation of bacteria

To isolate bacteria for 48 h, sampled swabs were cultured on sheep blood agar and selective medium (Katsukawa medium) at 35 °C, 5% $CO_2$ [18]. Katsukawa medium consists of heart infusion agar, 0·03% potassium tellurite (w/v), 10% sheep blood (v/v), and 0·05% activated charcoal (w/v) [18]. Samples were incubated for 18–24 h, after which colonies were transferred to dextrose sucrose starch (DSS) agar medium at 24, 30, and 48 h [19] to assess glucose and sucrose fermentation. The isolates, which were positive for glucose fermentation and negative for sucrose fermentation, were then characterized by Gram staining and evaluated using the catalase and urease tests. All Gram-positive organisms that were positive for catalase and urease production were suspected to be *C. ulcerans*. The isolates were biotyped using an API Coryne kit (bioMérieux, France) according to the manufacturer's instructions.

### Detection of tox gene by polymerase chain reaction (PCR)

To detect the *tox* gene in *C. ulcerans*, bacterial DNA templates were purified from the colonies and PCR analysis was performed as described previously [5, 20, 21]. The resulting product were purified and directly sequenced on an automated sequencer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Japan) using the primer pairs as described above.

### Elek immunoprecipitation test

The Elek immunoprecipitation test was performed using the antitoxin-in-well procedure to determine the exotoxin production of the isolates as described previously [20]. In brief, a central well (5 mm diameter) was prepared in an agar plate using a sterile stainless-steel blade followed by aspiration of the excised agar. The well was filled with 9 $\mu$l of standard diphtheria antitoxin (4·5 IU) (kindly provided by the National Institute of Infectious Diseases, Japan) and then surrounded by *C. ulcerans* strains that were stabbed into the agar at a distance of 10 mm from

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Table 2. *Characteristics of* Corynebacterium ulcerans *isolates*

| | | Isolation media | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Isolate no. | BA | K culture | DSS culture | Urea splitting | API Coryne (API number) | *tox* PCR | Elek |
| 1 | 2009–081 | + | + | W/B– | + | 0011326 | + | + |
| 2 | 2010–018 | + | + | W/B– | + | 0111326 | + | + |

DSS, Dextrose sucrose starch; PCR, polymerase chain reaction.

the edge of the centre well. *C. diphtheriae* PW8 was used as a positive control. The precipitation lines formed were observed after incubation for 1–2 days at 37 °C.

### Cytotoxicity assay for diphtheria toxin

Cytotoxicity assays for diphtheria toxin were performed using Vero cells to determine the exotoxin production of the isolates as described previously [20]. In brief, the liquid that condensed at the base of the Loeffler slant (Loeffler condensation) was collected after cultivating the *C. ulcerans* isolates for 3 days. The samples were filtered, and aliquots (25 $\mu$l) of the sample were added to Vero cells seeded in culture plates. The cytotoxic effect was determined 4 days after inoculation. To confirm the cytotoxic effect of diphtheria toxin, aliquots were mixed with a standard antitoxin (1 IU/ml) and added to Vero cell cultures after incubation at 37 °C for 30 min. The neutralization endpoints were determined 4 days after inoculation.

### Antibiotic susceptibility testing

Antibiotic susceptibility tests were conducted by the broth microdilution method using a Dry Plate DP24 (Eiken Chemical Co. Ltd). We used the following 16 antibiotics: benzylpenicillin (PCG), ampicillin (ABPC), cefazolin (CEZ), cefotiam (CTM), cefotaxime (CTX), cefaclor (CCL), cefditoren (CDTR), flomoxef (FMOX), imipenem (IPM), meropenem (MEPM), erythromycin (EM), clindamycin (CLDM), minocycline (MINO), vancomycin (VCM), levofloxacin (LVFX), and sulfamethoxazole–trimethoprim (ST). Sensitivity was assessed according to the Clinical and Laboratory Standards Institute's (CLSI) standard (M45-A) for *Corynebacterium* species. The sensitivity of the isolates to the eight drugs, for which CLSI standard values were not available was referenced from the data for similar drugs as follows: PCG was a reference for ABPC; CTX was a reference for CEZ, CTM, CCL, CDTR, and FMOX; TC was a reference for MINO; and CPFX was a reference for LVFX.

## RESULTS

*C. ulcerans* isolates were detected in two of the 37 domestic cats. The infected cats were relatively young, one was a 3-year-old male (no. 7) and the other was a 2-year-old female (no. 32). The isolates showed similar characteristics to those of *C. ulcerans*, including growth in sheep blood agar and Katsukawa medium, and DSS medium and positivity for API Coryne tests. Consequently, these isolates designated 0011326 and 0111326 as profiling numbers were identified as *C. ulcerans*. They were identified as toxigenic *C. ulcerans* by PCR, Elek test, and cytotoxicity assay (Table 2). The 2009-81 and 2010-18 isolates were susceptible to PCG, ABPC, CEZ, CTM, CTX, CCL, CDTR, FMOX, IPM, MEPM, EM, MINO, VCM, LVFX, and ST. The 2010-18 isolate was found to be resistant to CLDM (Table 3). The infected cats were kept indoors; however, they were allowed to roam freely outside (Table 4).

## DISCUSSION

In the present study, we surveyed 37 domestic cats and detected toxigenic *C. ulcerans* from two animals. This result, in addition to previous findings, suggests that toxigenic *C. ulcerans* is distributed over a wide area in Japan [12–14, 22]. In a cat infected with *C. ulcerans*, treatment with a steroid followed by antibiotics showed improvement. The isolates detected in the present study showed susceptibility to most of the drugs by antibiotic susceptibility testing, including antibiotics being used as treatments for the cats; however, drug treatments alone were insufficient for improvement of symptoms. This steroid appears effective for treating severe nasal obstruction, as reported in a previous study [22]. It is believed that

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Table 3. *Antibiotic susceptiblity findings for* Corynebacterium ulcerans *(broth microdilution method)*

| | Criteria (CLSI) | | | *C. ulcerans* isolates | |
|---|---|---|---|---|---|
| Drugs | S | I | R | No. 2009–081 | No. 2010–018 |
| PCG[a,b] | ≦1 | 2 | ≧4 | 0·12 | 0·12 |
| MPIPC | ≦2[c] | | ≧4[c] | | |
| ABPC[b] | ≦0·25[c] | | ≧0·5[c] | ≦0·25 | ≦0·12 |
| CEZ[b] | ≦8[c] | 16[c] | ≧32[c] | 0·25 | 0·25 |
| CTM[b] | | | | 0·5 | 0·5 |
| CTX[a,b] | ≦1 | 2 | ≧4 | 0·5 | 0·5 |
| CCL[b] | ≦8[c] | 16[c] | ≧32[c] | ≦0·5 | ≦0·5 |
| CDTR[b] | | | | 0·25 | 0·25 |
| CTRX[a] | ≦1 | 2 | ≧4 | | |
| CFPM[a] | ≦1 | 2 | ≧4 | | |
| FMOX[b] | ≦8[c] | 16–32[c] | ≧64[c] | 0·25 | ≦0·12 |
| IPM[a,b] | ≦4 | 8 | ≧16 | ≦0·06 | ≦0·06 |
| MEPM[a,b] | ≦4 | 8 | ≧16 | ≦0·06 | ≦0·06 |
| GM[a] | ≦4 | 8 | ≧16 | | 1 |
| ABK | | | | | |
| TC[a] | ≦4 | 8 | ≧16 | | 0·25 |
| DOXY[a] | ≦4 | 8 | ≧16 | | |
| MINO[b] | ≦4[c] | 8 | ≧16[c] | ≦0·25 | ≦0·25 |
| EM[a,b] | ≦0·5 | 1 | ≧2 | ≦0·06 | ≦0·06 |
| CLDM[a,b] | ≦0·5 | 1-2 | ≧4 | 0·25 | ≧4 |
| CP[b] | | | | | 4 |
| VCM[a,b] | 4 | – | – | 1 | 1 |
| TEIC | ≦8 | 16 | ≧32 | | |
| LZD[a] | 2 | – | – | | |
| Q/D[a] | ≦1 | 2 | ≧4 | | |
| FOM | | | | | |
| CPFX[a] | ≦1 | 2 | ≧4 | | ≦0·12 |
| LVFX[b] | ≦1[c] | 2[c] | ≧4[c] | ≦0·25 | 0·25 |
| ST[a,b] | ≦38/2 | | ≧76/4 | ≦4·74, 0·25 | ≦4·74, 0·25 |

[a] Assessments of sensitivities were determined according to the Clinical and Laboratory Standards Institute's (CLSI) standard (M45-A) for *Corynebacterium* spp.
[b] Antibiotic susceptibility tests were conducted by the broth microdilution method using a Dry Plate DP24 (Eiken Chemical Co. Ltd).
[c] Criteria for *Staphylococcus* spp.

improvement in this disease may be associated with other factors in cats such as the presence of another disease which decreases immunity or toxin production. Our results regarding antibiotic characteristics of the isolates, which had susceptibility to most of the drugs or CLDM only, are in agreement with previous studies [14, 22]. In addition, *Corynebacterium* spp. can be frequently isolated from the external ear or skin surface, and also from the nasal cavity of cats without any inflammation [26]. Therefore, there is a possibility that *C. ulcerans* could exist as part of the normal bacterial flora in cats, and the prevalence of toxigenic *C. ulcerans* is a result of differences in the environment or maintenance of the animals. Thus, epidemiological research is needed to clarify these issues in the future. In previous surveys it was reported that most infected cats were allowed to roam freely, or were stray animals. Our results are congruent with previous findings, and thus the infection can be due to activity outside of the home.

In our study, owners who kept the infected cats did not display any clinical symptoms, but cases of owners acquiring infections from their cats have been reported [15, 17, 23–25]. In some reports, owners with a weakened immune system, even if they did not have a disease, could be infected by *C. ulcerans*. In Japan, most infected people were aged >50 years, and these findings may be related to their antibody titres against diphtheria [24]. In other countries, injection of

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Table 4. *Summary of rearing environments of examined cats*

| Housing situation | No. of cats examined | Positive no. of *Corynebacterium ulcerans* |
|---|---|---|
| Indoors | 26 | 0 |
| Free roaming | 9 | 2 |
| Outdoors | 2 | 0 |
| Total | 37 | 2 |

diphtheria toxoid could decrease the severity of infection by toxigenic *C. ulcerans* [25]. It is thought that humans can generally be infected due to nasal catarrh and in some cases due to abscess or eye mucus [17, 26]. Therefore, it is necessary to investigate various bacterial infectious diseases in cats.

The number of patients with diphtheria in Japan reached 86000 in 1945. After that point infections decreased, and there have been no reports since, except for one case reported in 1999 [27]. The Ministry of Health, Labour and Welfare reported that effective vaccination enables infants to possess high levels of antibodies against diphtheria leading to a marked decrease in antibody prevalence rates in adults aged >45 years [27]. Effectiveness of vaccination for diphtheria has not been fully evaluated [16, 28, 29], and thus further epidemiological surveys of toxigenic *C. ulcerans* must be conducted to solve this controversial issue. Veterinarians should consider infection with *C. ulcerans* in cats with purulent disease, particularly upper respiratory tract diseases such as nasal catarrh. However, there are no inspection agencies in Japan to identify *C. ulcerans* or assess its toxin production. Therefore, it appears important to ensure there is cooperation between clinical sites and research institutions or develop simple assay methods. In the present study, we identified *C. ulcerans* by API Coryne. More recently, new methods such as partial RNA polymerase b-subunit (rpoB) sequencing or matrix-assisted laser desorption-ionization–time-of-flight mass spectroscopy (MALDI–TOF/MS) have been reported [30, 31]. For further analysis, these highly sensitive identifications could be useful in helping to control the infection.

In humans, animals play an important role in the community, and they are closely associated with human society. The risk of zoonosis from domestic animals to humans such as infection by *Brucella canis* [32] and *Capnocytophaga canimorsus* [33], in addition to toxigenic *C. ulcerans*, is increasing, but the pathogenicity of most microorganisms in animals remains unclear. Thus, veterinarians may struggle to explain the risks to owners or to treat the animals. In this study, we isolated toxigenic *C. ulcerans* from domestic cats with chronic rhinitis, and it is believed that this bacterium can cause respiratory tract infection in cats. The infected cats were kept indoors or allowed to roam freely outside, and they were probably in close contact with their owners. Thus, diseases generally found in popular animals could be contracted by humans.

Thus, systems for correct diagnosis and detailed examination of diseases are needed, and people must be educated to develop a healthy relationship between humans and animals. Furthermore, it is important to devise inspection systems or conduct epidemiological surveys in cooperation with veterinarians in animal hospitals and public health officials.

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## DECLARATION OF INTEREST

None.

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