bodies against both *F. tularensis* subsp. *tularensis* and subsp. *holarctica* in rabbit and mouse sera were detected by cELISA (Fig. 2b and c). Although we could not test convalescent human patients sera infected with *F. tularensis* subsp. *tularensis* due to unavailability in Japan, the result in the present study indicated that the cELISA is capable to detect antibodies induced after both *F. tularensis* subsp. *tularensis* and subsp. *holarctica* infections. On the other hand, when serum sample of a mouse immunized with *F. novicida* were tested, 11% inhibition was observed (Fig. 2c). This slight inhibition may be due to the common antigenic epitopes shared by *F. novicida* and *F. tularensis* since they are genetically closely related (29). In addition, no serum samples of rabbits immunized with *Brucella* spp. and *Yersinia* spp. interfered with the specific binding of MAb to LPS (Fig. 3). These results indicate that MAb-based cELISA is highly specific for *F. tularensis* without any cross-reactions with other microorganisms.

The results of cELISA of serum samples of tularemia patients and healthy donors were evaluated. ROC analysis showed that cELISA had sensitivities of 91.1 and 97.0% at cutoff values of 25.8% inhibition and 8.5% inhibition, respectively (Fig. 4). Two serum samples of patients at day 1 (P4) and day 11 (P1) after onset of symptoms were negative by cELISA at a cutoff value of 25.8%. These sera were also negative by iELISA and MA (Table 1), and thus it was conceivable that these acute-phase sera did not contain detectable levels of antibodies against *F. tularensis*. However, when the cutoff value was decreased to 8.5% inhibition, these two acute-phase sera became positive, indicating that cELISA with a cutoff value of 8.5% is more sensitive in detecting antibodies against *F. tularensis* than iELISA and MA. In contrast, serum sample of the patient (P17) obtained 8 months (241 days) after recovery was negative even at a cutoff value of 8.5% inhibition, although the serum was positive by iELISA and MA. The paired serum of the same patient (P17) obtained 2 weeks after disease onset was clearly positive by both cELISA and iELISA, but negative by MA. These results might suggest that LPS antibodies recognizing the specific epitope of the MAb M14B11 in patients did not persist for several months, whereas the antibodies recognizing the other epitopes on LPS persisted for more than 8 months. The testing of large numbers of serum samples from both acute and convalescent phases of tularemia patients might have improved the precise interpretation of data. For the 50 healthy human serum samples, cELISA exhibited 100% specificity at both cutoff values of 25.8 and 8.5% inhibition (Fig. 4c). Therefore, the cutoff value of 8.5% inhibition was considered to be suitable for sensitive and specific detection of *F. tularensis* antibodies in human serum samples based on the ROC analysis.

The sensitivity of cELISA and iELISA were comparable, whereas MA was less sensitive in detecting *F. tularensis* antibodies (Tables 2 and 3). All of these results were comparable to those of previously reported cELISA methods that detected antibodies against *F. tularensis* outer membrane protein antigens with a sensitivity and specificity of 95.7 and 96%, respectively (30). However, it is noteworthy that LPS is a more suitable antigen for detecting an antibody response elicited in the early phase after *F. tularensis* infection as well as antibodies that persist for several years (10, 24).

In the present study, we also found, in agreement with a previous report, that antibodies against *F. tularensis* persist for months after infection (16). Although we could not test how long the antibodies persist, a moderately decreasing antibody level was observed after 6 months (Fig. 6), and this pattern was the same in serum samples of two different patients with comparable antibody levels in cELISA and iELISA. Indeed, the correlation between cELISA and iELISA was high ($R^2 = 0.8226$) (Fig. 5). These data suggest that the results obtained for detection of antibodies against *F. tularensis* by the novel cELISA are reliable and consistent with those obtained by iELISA.

Our cELISA method based on the inhibition of binding of MAb specific to the *F. tularensis* LPS is highly sensitive and specific. This method is well suited as a routine, confirmatory laboratory test for tularemia in humans. It can overcome the problems associated with conventional serological assays such as low sensitivity and specificity in MA or requirements of species-specific secondary antibodies in iELISA or indirect immunofluorescence assay. Therefore, our MAb-based cELISA is expected to be advantageous and useful for serosurveillance of various wild animals such as bears, wild boars, fox, raccoon dogs, and even birds, except for the highly susceptible animals such as some species of rodents or lagomorphs since they die before producing antibodies to *F. tularensis*. A seroepidemiological study of various wild animals with this novel cELISA is ongoing. We believe this cELISA method will help facilitate the surveillance of tularemia in humans and wild animals, leading to better understanding of the ecology and epidemiology of tularemia.

ACKNOWLEDGMENTS

We thank H. Fujita, Ohara Research Laboratories, Ohara General Hospital, Fukushima, Japan, for providing serum samples of patients and healthy blood donors. We also thank K. Imaoka (Department of Veterinary Science, NIID) for providing rabbit sera immunized with *Brucella* spp. and *Yersinia* spp.

This study was supported in part by Health and Labor Science Research Grants for Research on Emerging and Re-Emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare in Japan (H22-Shinkou-Ippan-010). N.S. was supported by a Tokyu Foundation Scholarship for Inbound Students.

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Sekizuka et al. BMC Microbiology 2012, 12:72
http://www.biomedcentral.com/1471-2180/12/72

BMC Microbiology

RESEARCH ARTICLE Open Access

Corynebacterium ulcerans 0102 carries the gene encoding diphtheria toxin on a prophage different from the *C. diphtheriae* NCTC 13129 prophage

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Abstract

Background: *Corynebacterium ulcerans* can cause a diphtheria-like illness, especially when the bacterium is lysogenized with a *tox* gene-carrying bacteriophage that produces diphtheria toxin. Acquisition of toxigenicity upon phage lysogenization is a common feature of *C. ulcerans* and *C. diphtheriae*. However, because of a lack of *C. ulcerans* genome information, a detailed comparison of prophages has not been possible between these two clinically important and closely related bacterial species.

Results: We determined the whole genome sequence of the toxigenic *C. ulcerans* 0102 isolated in Japan. The genomic sequence showed a striking similarity with that of *Corynebacterium pseudotuberculosis* and, to a lesser extent, with that of *C. diphtheriae*. The 0102 genome contained three distinct prophages. One of these, ΦCULC0102-I, was a *tox*-positive prophage containing genes in the same structural order as for *tox*-positive *C. diphtheriae* prophages. However, the primary structures of the individual genes involved in the phage machinery showed little homology between the two counterparts.

Conclusion: Taken together, these results suggest that the *tox*-positive prophage in this strain of *C. ulcerans* has a distinct origin from that of *C. diphtheriae* NCTC 13129.

Keywords: Bacteriophage, Toxin gene, Horizontal gene transfer, Diphtheria, Zoonosis

Background

A diphtheria-like infectious disease caused by *Corynebacterium ulcerans* is increasing in clinical importance in developed countries and is now regarded as "diphtheria" in Europe [1,2]. Infection with *C. ulcerans* occurs in a wide range of hosts, including cats, dogs, pigs, cows, and whales [3-9]. The first clearly documented case of zoonotic transmission involved a dog, as reported by Lartigue et al. [5]. This is in contrast to the causative agent of classical diphtheria, *C. diphtheriae*, whose host species is thought to be limited to humans [10]. Nevertheless, the two species share a common feature: upon lysogenization of *tox*-encoding bacteriophages, they become toxigenic and are able to produce the potent diphtheria toxin [1,10]. This toxin is known to contribute to disease progression, occasionally leading to death. It is encoded by a single gene designated *tox*, situated inside prophages lysogenized in the bacterial genome of *C. diphtheriae* [11]. The prophages are capable of induction, by ultraviolet light or DNA-damaging agents such as mitomycin C, and yield β-, δ-, ω- and other functional bacteriophage particles [12]. Some types of bacteriophages can infect both *C. diphtheriae* and *C. ulcerans* [13-16]. Furthermore, the *C. ulcerans tox* gene is also encoded in a genome region surrounded by phage attachment (*att*) sites conserved between the two species [7,16]. The nucleotide sequences of *C. ulcerans tox* genes were published by Sing et al. They showed some

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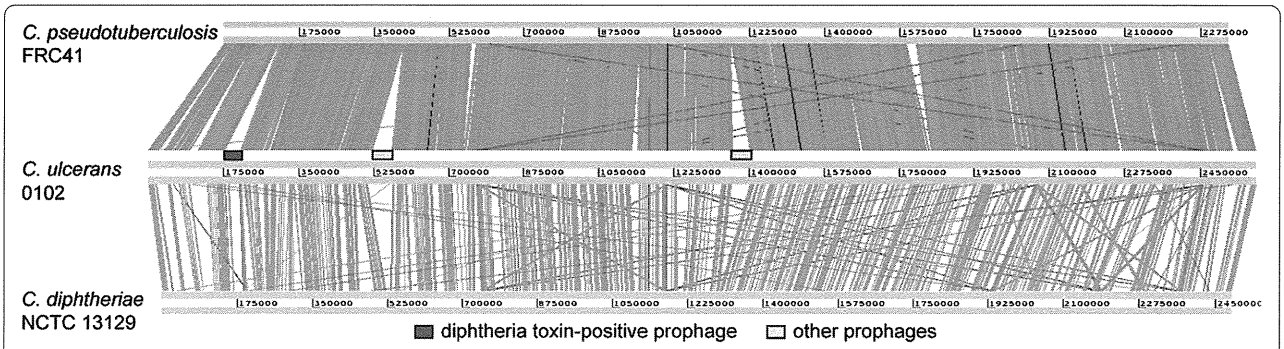

Figure 1 Schematic genome comparison. *C. ulcerans* 0102 (middle) with *C. pseudotuberculosis* FRC41 (top) and *C. diphtheriae* NCTC 13129 (bottom) using a BLASTN homology search visualized by the ACT program. The red and blue bars between chromosomal DNA sequences represent individual nucleotide matches in the forward and reverse directions, respectively. BLASTN match scores less than 200 are not shown. A blue box and two yellow boxes represent a *tox*-positive prophage and other prophages on the chromosome of *C. ulcerans* 0102, respectively.

diversity in the genetic sequence among *C. ulcerans* strains, in contrast to the highly conserved *C. diphtheriae tox* gene [17,18].

In 2003, the nucleotide sequence of the whole genome of *C. diphtheriae* strain NCTC13129 was reported [19]. The sequence information revealed some striking features of the bacterial genome, such as the presence of as many as 13 pathogenicity islands (PAIs) [19], uncommon among *C. diphtheriae* strains [20]. The presence of a *tox*-positive prophage flanked by the *att* regions was confirmed and supported the findings of previous reports [21]. Despite comparable clinical importance, the genomic sequence of toxigenic *C. ulcerans* has not yet been reported. In the present study, we determined the nucleotide sequence of the toxigenic *C. ulcerans* isolate 0102 genome, obtained in 2001 from the pharyngeal pseudomembrane of a 52-year-old woman presenting with a sore throat and fever. This was the first toxigenic *C. ulcerans* infection reported in Japan. This patient had been living with nearly 20 cats before the onset of illness [22]. Details of the bacteriological characteristics of the isolate have been described elsewhere [23]. Our analysis was especially directed towards the structure of the *tox*-positive prophage because of its unexpectedly novel structure.

Results

Genome sequence and genomic information for *C. ulcerans* 0102

To determine the complete genome sequence of *C. ulcerans* 0102, obtained short reads were assembled into five contigs by *de novo* assembly. Each gap was filled by direct PCR and sequencing. A circular chromosome sequence of *C. ulcerans* 0102 represents 2,579,188 bp, with a G + C content of 53.4% (Additional file 1) and corresponds to the predicted restriction fragment profiles obtained by PFGE analysis (Additional file 2). The chromosome possesses 2,349 coding sequences, 51 tRNA genes, and 4 *rrn* rRNA operons.

Comparative genome analysis of three pathogenic *Corynebacterium* spp

Pair-wise sequence alignment revealed a highly conserved synteny among pathogenic *Corynebacterium* spp. (*C. pseudotuberculosis* FRC41, *C. ulcerans* 0102, and *C. diphtheriae* NCTC 13129; Figure 1). No significant genome rearrangements, such as inversion or transposition events, were observed among the three species, in accordance with previous findings [24]. The sequence similarity suggests that the chromosomes of *C. ulcerans* 0102 and *C. pseudotuberculosis* FRC41 are highly similar compared with that of *C. diphtheriae* NCTC 13129 (Figure 1). Once again, this is in accordance with previous findings in other *C. ulcerans* strains [24]. Similarly, a neighbor-joining phylogenetic tree, based on the partial sequence of *rpoB*, indicates that *C. ulcerans* 0102 is closely related with *C. pseudotuberculosis*, but clearly distinguishable from the *C. diphtheriae* clade (Additional file 3). Three prophages, ΦCULC0102-I, -II, -III, were identified in *C. ulcerans* 0102. One of the prophages, ΦCULC0102-I, carries *tox*, the gene encoding the diphtheria toxin (Figure 1).

The *tox*-positive prophage of *C. ulcerans* 0102

The ΦCULC0102-I prophage of *C. ulcerans* 0102 is integrated into $tRNA^{Arg}$ (CULC0102_t08) (Figure 2), suggesting that the integration site is identical to that in the *C. diphtheriae* NCTC 13129 corynephage. In contrast, the recently reported *C. ulcerans* 809 and *C. pseudotuberculosis* FRC41 genomes possess a phage-related integrase (*intC*) and a nitric oxide reductase (*nor*) gene, respectively, instead of a prophage (Figure 2). Putative attachment sequences were similar between both prophages carrying the *tox* genes (Additional file 4).

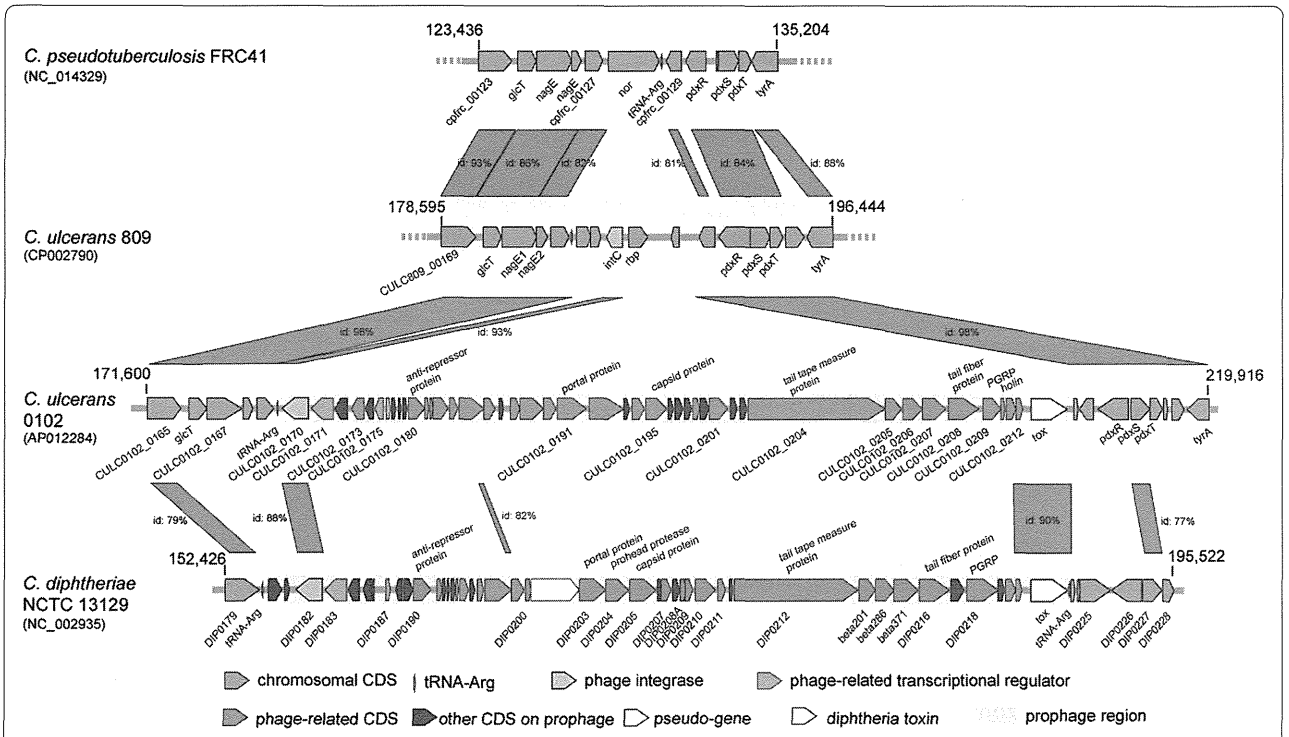

Figure 2 Schematic representation and comparative analysis of *tox*-positive prophages and flanking regions. The *tox*-positive prophage and flanking regions of *C. ulcerans* 0102 and *C. diphtheriae* NCTC13129 are shown. The corresponding region of *C. pseudotuberculosis* FRC41 and *C. ulcerans 809* is also shown. Boxes indicate individual coding regions with colors assigned to their functions. GenBank accession numbers are given in parentheses.

The two *tox*-positive prophages share the same structural features, with genes aligned in an 'integrase - packaging - head - tail - lysis - toxin' orientation (Figure 2). Pair-wise alignment of the prophages indicates a high similarity in the region encoding the putative integrase, the 3′-ends of CULC0102_0211 and CULC0102_0212, *tox*, and the attachment sites (Figure 2). The major phage machineries encoded in the internal phage region showed low similarity at the nucleotide and amino acid levels (less than 18%) between *C. ulcerans* 0102 and *C. diphtheriae* NCTC13129.

Discussion

Whole-genome sequencing has revealed that the *C. ulcerans* 0102 genome is composed of 2,579,188 bp with a G + C content of 53.4%. These values are similar to those recently reported for *C. ulcerans* strains 809 (2,502,095 bp, 53.3% G + C) and BR-AD22 (2,606,374 bp, 53.4% G + C) [24]. *C. ulcerans* 0102 shares many common features with the two previously reported strains, including 12 virulence factors. Strain 0102 is distinctive with respect to the features of prophages integrated in its genome. It possesses a unique *tox*-positive prophage, ΦCULC0102-I, in its chromosome (Figure 1 and Additional file 1). In the same position of the recently reported *C. ulcerans* 809 genome exists a remnant phage-related integrase (*intC*) gene [24] (Figure 2). The *C. ulcerans* 0102 prophage differs from the corresponding prophage in *C. diphtheriae*. Although the integrase and *tox* gene sequences of ΦCULC0102-I showed high similarity to those of the corynephage encoding *tox* in *C. diphtheriae* NCTC 13129, the major phage machinery genes in ΦCULC0102-I are distinct from those in other corynephages in *C. diphtheriae* (Figure 2). This suggests that *C. ulcerans* 0102 did not immediately acquire the *C. diphtheriae tox*-positive corynephage.

There are many possible explanations for the origins of these two prophages that are *tox*-positive but obviously different. One of the simplest explanations we can postulate is outlined in Figure 3. Generally, bacterial prophages are duplicated by excision from chromosomal DNA and subsequent concatenation at both ends of the *att* sites (Figure 3A). This duplication step indicates that two highly homologous regions, *int* and *tox*, could be in close proximity and adjacent to the *att* site concatenation. It could be speculated that homologous recombination between two prophages may facilitate the acquisition of the *tox* gene in *C. ulcerans* 0102 from an unknown *tox*-positive prophage (Figure 3B) [25]. Horizontal gene transfer is one of the major mechanisms of foreign gene acquisition by bacteria, as reviewed by Ochman et al. [26]. Liu et al. have

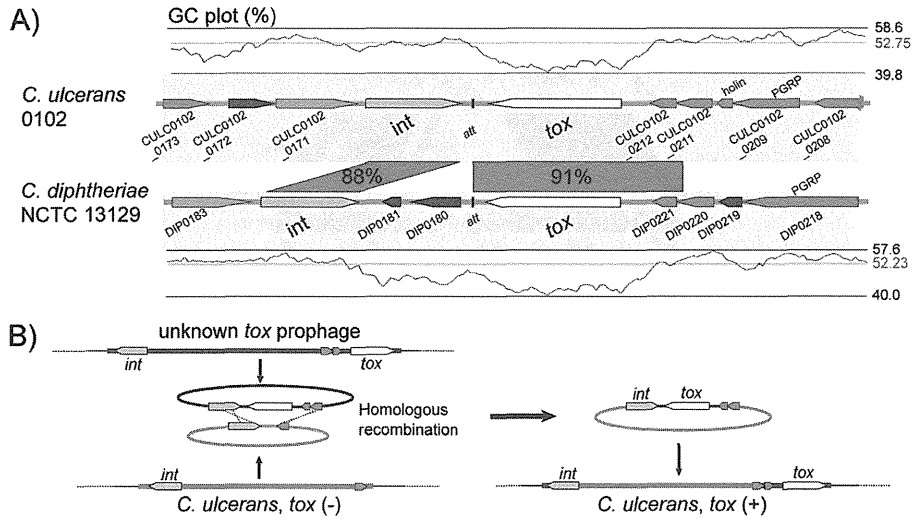

Figure 3 Schema of the diphtheria toxin acquisition hypothesis. (A) Pair-wise comparison of regions with high similarity between *C. ulcerans* and *C. diphtheriae*. These structures of putative phages are constructed by connecting attachment sites. The plots above and below represent the GC content calculated with a window size of 500 bp. **(B)** Schematic representation of how diphtheria toxin has been acquired in *C. ulcerans*.

demonstrated that horizontally transferred genes are often disabled and become pseudogenes. In these cases the genes are no longer beneficial to the recipients [27]. Non-toxigenic *C. diphtheriae* (CD450, CD119, CD448, and CD443 strains) carry *tox* pseudogenes that are relatively similar to the *tox* genes of *C. ulcerans* (Additional file 5), suggesting that horizontal gene transfer among *Corynebacterium* spp. might occur. Consistent with previous findings [7,17,18,28], tthe *tox* gene in *C. ulcerans* 0102 is not identical to that of *C. diphtheriae* (Additional file 5); phylogenetic analysis of *tox* showed greater heterogeneity among *C. ulcerans* isolates than that for *C. diphtheriae* isolates (Additional file 5).

The *C. diphtheriae tox* gene is highly conserved among temporally and geographically diverse strains [29], therefore greater variation in *tox* genes from *C. ulcerans* isolates suggests that this strain might have acquired the *tox* gene before *C. diphtheriae*.

In a recent report, whole genome sequence analysis of non-toxigenic *C. ulcerans* 809 and BR-AD22 [24], the β-corynephage-like truncated integrases (CULC809_00176 and CULC22_00173) are located adjacent to the $tRNA^{Arg}$ gene, similar to ΦCULC0102-I in *C. ulcerans* 0102 and *C. diphtheriae*. The $tRNA^{Arg}$ gene (CULC0102_t08) appears to be a 'hotspot' for the acquisition of ΦCULC0102-I-like prophages by homologous integrase.

The whole genome sequences of *C. ulcerans* 809 and BR-AD22 contain possible virulence factors, such as corynebacterial protease (CP40), phospholipase D (Pld), neuraminidase (NanH), venom serine protease (Vsp1), trypsin-like serine protease (TspA), Rpf interacting protein (RpfI), cell wall-associated hydrolase (CwlH), and five surface-anchored proteins (SpaB–F) [24]. The SpaA-type pilin, encoded by the *spaABC-srtA* gene cluster, is considered to play a crucial role in adhesion of *C. diphtheriae* [30]. The gene encoding the shaft protein of SpaA-type pilin (*spaA*) was absent in *C. ulcerans* 0102, a feature consistent with previous findings in *C. ulcerans* 809 and BR-AD2 [24]. As SpaB and SpaC proteins, which are assumed to be present in all three *C. ulcerans* strains, can contribute to host-cell adhesion in the absence of SpaA [30], this may imply a common mechanism of cell adhesion by *C. ulcerans* [24].

The *C. ulcerans* 809 strain was isolated from a patient with a rapid fatal pulmonary infection. The 809 strain-unique virulence factor (shiga toxin-like ribosome-binding protein, Rbp) is located adjacent to the truncated integrase (CULC809_00176) and corresponds to the integrase of ΦCULC0102-I. It appears that virulence factors have been acquired as a cassette gene in the ΦCULC0102-I-like prophage. It is intriguing to note that the 0102 strain does not carry the 809 strain-unique virulence factors (Rbp and the additional venom serine protease, Vsp2), but instead carries the *tox* gene on ΦCULC0102-I, which resulted in a diphtheria-like illness in a 52-year-old woman.

Isolates of *C. ulcerans* are generally obtained from a diverse range of animals, including humans. Isolation of a human pathogen *C. diphtheriae* from animals has been reported previously, although it is rare [31]. The *tox* gene might be frequently transmitted through common

prophages with the aid of the highly homologous regions among *Corynebacterium* spp., including *C. diphtheriae* and *C. ulcerans* isolated from animal sources.

Conclusions

Toxigenic *C. ulcerans* is an emerging pathogen that can be transmitted from animals to humans [5]. In the host organism, as well as in *C. diphtheriae*, the *tox* gene [18] is encoded by prophages. Through genome sequencing, we have identified a novel structure in a *tox*-positive *C. ulcerans* prophage with no significant sequence homology to those in *C. diphtheriae*. This suggests distinct origins of the prophages and thus may also explain the difference in the primary structures of their *tox* genes. The *tox*-positive bacteriophages may increase the dissemination risk of toxigenic *C. ulcerans* isolates, therefore, *C. ulcerans* isolates from both human and animal sources should be investigated further to determine the level of variation.

Methods

This research was not carried out on humans. No experimental research on animals was carried out.

Bacterial strain

The toxigenic *C. ulcerans* isolate 0102 was obtained in 2001 as a human clinical isolate [22,23].

Preparation of genomic DNA

Genomic DNA was isolated by conventional methods, using phenol extraction and ethanol precipitation from heat-killed bacterial cells propagated in brain-heart infusion liquid medium.

Short-read DNA sequencing using an Illumina Genome Analyzer IIx

DNA libraries of the ~600 bp insert length of *C. ulcerans* 0102 were prepared using a genomic DNA Sample Prep Kit (Illumina, San Diego, CA, USA). DNA clusters were generated on a slide using a Cluster Generation Kit (ver. 4) on an Illumina Cluster Station (Illumina), according to the manufacturer's instructions. Sequencing runs for 80-mer short reads were performed using an Illumina Genome Analyzer IIx (GA IIx) and TruSeq SBS kit v5. Fluorescent images were analyzed using the Illumina base-calling pipeline RTA2.6/SCS2.8 to obtain FASTQ-formatted sequence data.

De novo assembly of short DNA reads and gap-closing

The 80-mer reads were assembled (parameters k64, n51, c32.1373) using ABySS-pe v1.2.0 [32]. Predicted gaps were amplified with a specific PCR primer pair, followed by Sanger DNA sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Validation of the complete genome sequence using short-read mapping and pulsed-field gel electrophoresis (PFGE)

To validate the genome sequence, 40–mer short reads were re-aligned with the sequence using Maq software (ver. 0.7.1) and the *easyrun* Perl-command [33]. Read alignment was inspected using the MapView graphical alignment viewer [34]. PFGE analysis was performed to validate the predicted restriction fragment profiles from the complete genome sequence, according to De Zoysa et al. [35]. Bacterial cells were lysed with lysozyme and protease [36], embedded in plugs, digested with the restriction endonuclease *SfiI* (New England Biolabs, Ipswitch, MA, USA) and electrophoresed in a CHEF DRII apparatus (Bio-Rad, Hercules, CA, USA) at 11°C with a pulse time of 5–20 s for the first 20 h and 1–5 s for the following 18 h.

Annotation and pair-wise alignment analysis

Gene prediction from the complete sequence was performed using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; http://www.ncbi.nlm.nih.gov/genomes/static/pipeline.html). Several of the suggested errors were revised manually. Pseudogenes that were identified by PGAAP were checked using the read-mapping correction described above. Genomic information, such as nucleic acid variations and circular representation, was analyzed using IMC-GE software (Insilicobiology, Yokohama, Japan). A BLASTN homology search [37] was performed for the whole chromosome sequences of *C. pseudotuberculosis* FRC41 (accession no. NC_014329), *C. ulcerans* 0102, and *C. diphtheriae* NCTC 13129 (accession no. NC_002935). Aligned images of the homologous regions were visualized with the ACT program [38].

Phylogenetic analysis

Phylogenetic analyses of all nucleotide sequences were conducted using the neighbor-joining method with 1,000-times bootstrapping in ClustalW2 [39]. FigTree ver. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) software was used to display the generated tree.

Nucleotide sequence accession numbers

The complete chromosome sequence for the *C. ulcerans* 0102 strain has been deposited in the DNA Data Bank of Japan (DDBJ; accession no. AP012284).

Additional files

Additional file: 1 Circular representation of the *C. ulcerans* 0102 genome. From the outside inward, the outer circle 1 indicates the size in base pairs (Mb). The red bars on Circle 2 show prophage region. Circles 3 and 4 show the positions of CDS transcribed in clockwise and anticlockwise directions, respectively. The dark blue bars on circle 5 indicate ribosomal DNA loci. Circle 6 shows a plot of G + C content (in a 20 kb window). Circle 7 shows a plot of GC skew ([G - C]/[G + C]; in a 20 kb window).

Additional file: 2 PFGE analysis of *C. ulcerans* 0102 with four restriction enzyme digestions.

Additional file: 3 Jukes-Cantor-derived phylogenetic tree based on the partial *rpoß* gene region among *Corynebacterium* isolates with 1,000-fold bootstrapping. Scale bar indicates number of substitutions per site. The number at each branch node represents the bootstrapping value. GenBank accession nos. given in parentheses.

Additional file: 4 Alignment of the nucleotide sequences of attachment site common regions among *C. ulcerans* 0102 and *C. diphtheriae* NCTC 13129. The red characters show regions annotated as $tRNA^{Arg}$.

Additional file: 5 Phylogenetic tree based on the tox genes among toxgenic and nontoxigenic *Corynebacterium* spp. using the Neighbor-joining method with 1,000-fold bootstrapping. Scale bar indicates number of substitutions per site. The number at each branch node represents the bootstrapping value. GenBank accession nos. given in parentheses.

Competing interests
The authors declare that they have no competing interests.

Acknowledgments
The authors are grateful to Akio Hatanaka, Atsuhiro Tsunoda and Kenji Ooe for the 0102 clinical isolate. This work was supported by grants for Research on Emerging and Re-emerging Infectious Diseases (H23 Shinko-Ippan-007 and H22-Shinko-Ippan-010), from the Ministry of Health, Labour and Welfare, Japan.

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Authors' contributions
TS and FT carried out the genome sequencing studies, participated in the sequence alignment and drafted the manuscript. TKo carried out maintenance, quality control and propagation of the bacterial strain for genome sequencing. AY and TKe participated in the design of the study. MT and KS conceived of and participated in coordination of the study, respectively. MK and MI coordinated the study, and drafted and finalized the manuscript. All authors read and approved the final manuscript.

Received: 10 January 2012 Accepted: 14 May 2012
Published: 14 May 2012

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doi:10.1186/1471-2180-12-72
Cite this article as: Sekizuka *et al.*: *Corynebacterium ulcerans* 0102 carries the gene encoding diphtheria toxin on a prophage different from the *C. diphtheriae* NCTC 13129 prophage. *BMC Microbiology* 2012 12:72.

日耳鼻 115：682-686，2012

症例報告

茨城県で発見された コリネバクテリウム・ウルセランスの１症例

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飼い猫からジフテリア毒素産生性の *Corynebacterium ulcerans* に感染した症例を経験した．症例は51歳女性，難治性の咽頭痛を主訴に来院し，上咽頭後壁の厚い偽膜形成と黄色膿汁が認められた．理学所見と患者の動物飼育歴より *C. ulcerans* 感染症の可能性を考え，マクロライド系抗菌薬にて治療したところ症状は速やかに改善した．患者の咽頭の白苔および飼い猫の眼脂より *C. ulcerans* を検出し咽頭炎の起炎菌ならびに感染経路を確定することができた．*C. ulcerans* は人獣共通感染症を引き起こす菌の一つであり，本邦では本報告を含めて飼い猫からの感染例が数例報告されている．偽膜性咽頭炎の鑑別診断には必ず *C. ulcerans* 感染症を考慮し診察することが重要である．

キーワード：コリネバクテリウム・ウルセランス，人獣共通感染症
急性咽頭炎

はじめに

ジフテリア毒素を産生するコリネバクテリウム・ウルセランス（以下 *C. ulcerans*）は，人獣共通感染症を起こす細菌であり，英国をはじめとした欧州諸国では，ジフテリア症類似の臨床所見を呈する感染症として以前から問題になっている．2001年の千葉県での発生例以来，本邦でも *C. ulcerans* によるヒト感染例が散見されるようになった．また，本邦ではペットからの感染が疑われるような報告が多い．今回われわれは茨城県内で初めて確認された *C. ulcerans* 感染症を経験した．本症例では発症前にペットである猫が感冒様症状を呈しており，この猫の眼脂から *C. ulcerans* が検出されたため感染経路を特定することが可能であった．症例を報告するとともに，日本における *C. ulcerans* 感染の現状とその背景因子について検討したので報告する．

症　例

患者：51歳女性

既往歴：特記事項無し．ジフテリアワクチン歴は不明

家族歴：特記事項無し

生活歴：屋外に自由に出入りする猫を１匹飼育中

現病歴ならびに治療経過：2010年９月中旬より咽頭痛，嚥下時痛を自覚した．発症２日目に内科を受診し，経口抗菌薬（CDTR-PI）の内服を開始するも改善がみられなかった．発症９日目に近くの耳鼻咽喉科を受診したところ上咽頭に白色偽膜が認められ，同日当科に紹介，急性上咽頭炎の診断で緊急入院となった．初診時，上咽頭から中咽頭に厚く付着する白色の偽膜が認められた（図１）．入院当日よりABPC-ST 4.5g/dayの経静脈投与を開始した．入院翌日，患者の生活歴（ペット飼育歴）より *C. ulcerans* 感染症を疑ったためEM 1g/dayの経静脈投与に変更した．その結果，自覚症状ならびに上咽頭所見は速やかに改善を示した．しかし，患者が徐々にEM静注後に気分不快を訴えるようになり，第４病日よりCAM 400mg/dayの経口内服に変更した．第５病日には上咽頭所見はほぼ正常化し，第７病日退院となった．退院後もCAM 400mg/dayの経口投与を１週間継続し，症状は完全に消失した．その後は，８カ月の間再発を認めていない．初診時に提出した上咽頭偽膜からの培養よりグラム陽性桿菌が検出された．初診時より14日目に，菌は国立感染症研究所の細菌学的検査により起因菌は，*C. ulcerans* と同定され，さらにその２日後にジフテリア毒素を産生する *C. ulcerans* であることが確認された．患者にペットに関する詳しい問診を行ったところ，症状発現のおよそ１週間前に飼い猫が膿性鼻汁を伴う上気道感染に罹患していたことが判明した．そのため，飼い主である患者より承諾を得て飼い猫からの菌検査を行ったところ眼脂から *C. ulcerans* が検出された．

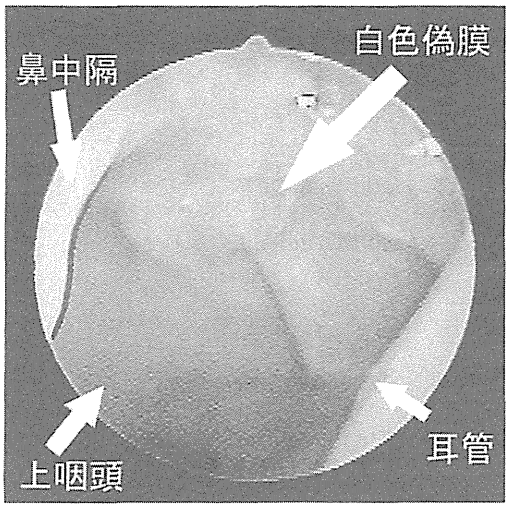

図1　来院時咽頭所見
上咽頭に偽膜形成を認める．

細菌学的検査：患者およびネコから分離された *C. ulcerans* の毒素原性をPCR法，Elek試験法，培養細胞法で試験した．その結果，すべての方法でジフテリア毒素の産生能が確認された．

考　　察

C. ulcerans は，1928年にGilbertとStewartによって発見された人獣共通感染症を起こすグラム陽性桿菌である[1]．ヒトにはジフテリア症状を来すことが海外では比較的よく知られている[2]．宿主は哺乳類の家畜などから，野良犬，野良猫に加え，鳥類であるペンギンでの感染例も報告されており，ヒトも含めてかなり幅広い動物を宿主とする細菌である[3][4][5]．*C. ulcerans* がヒトで感染を起こすと，咽喉頭の典型的なジフテリア様症状のみならず，皮膚炎，頸部リンパ節炎，腹膜炎など多彩な症状を呈することがある．本邦での初報告例などのように古典的ジフテリア症状を来すものもある[6]．古典的ジフテリア症例では，ジフテリア毒素の神経障害のため不整脈を生じることがあるとされているが，われわれが渉猟しえた範囲では，*C. ulcerans* 感染例での不整脈の報告はみられなかった．海外でのヒト感染経路は牛，羊等との接触や，非加熱処理の乳製品摂取によるものが多いとされているが，愛玩動物からの感染報告もみられる[7]．典型的と思われる厚い白色偽膜の咽喉頭所見を呈する感染症の鑑別診断として，溶連菌感染症，伝染性単核球症，古典的ジフテリア症，単純ヘルペスなどが挙げられる．今回の症例は厚く乳白色の偽膜の存在と患者のペット飼育歴から，*C. ulcerans* 感染症を疑い診断に至った．本疾患においては感染経路に関する問診も重要であり，ペット飼育歴があり，特に感冒症状を来したペットへの接触が存在した場合には，詳細な細菌学的検査など含め精査を進める必要がある．通常の細菌同定キットで *C. ulcerans* の同定はできないため，*Corynebacterium* 用の同定キットの使用が推奨される．より迅速な診断のためには，米国ではスクリーニング目的にPCR法による毒素遺伝子の同定または発現の確認が推奨されている[8]．感染経路が不明な例もあるが，ヒトからヒトへの感染や，院内感染などはこれまでのところ報告されていない．治療については，おおむね抗菌薬に対する感受性は良好とされているが，われわれの経験からはβラクタム系抗菌薬はあまり有効でなく，マクロライド系抗菌薬が有効であった．また，*C. diphteriae* が産生するジフテリア毒素は心筋の神経伝達を障害する可能性があり，不整脈が生じたような重症例では抗ジフテリア毒素血清の使用が望ましいと考えられるが，*C. ulcerans* が産生するジフテリア毒素では不整脈が起きた事例の報告は現在までにはない．通常 *C. ulcerans* にはジフテリア毒素産生能はなく牛や馬の正常細菌叢として機能するものとされている．しかし，これまで国内で発見，報告された8症例に共通して，同定されたすべての *C. ulcerans* はジフテリア毒素産生能が認められた．*C. ulcerans* の産生する毒素は，*C. diphteriae* の産生毒素やトキソイドで免疫した血清で中和される．国内で分離された *C. ulcerans* の症例はすべてジフテリア毒素産生性（*C. ulcerans* と *C. diphteriae* のジフテリア毒素遺伝子はアミノ酸配列の解析により異なることが判明している[9]）で，その病因はジフテリア毒素に起因すると考えられている．さらに興味深い点として，報告されている全症例が50歳代で発症していた点が挙げられる．8例中3例では，飼い猫からの感染が強く疑われており，それ以外の症例でも動物からの感染が疑われる．東京都や厚生労働省健康局結核感染症課と国立感染症研究所感染症情報センターで5年ごとに行っているジフテリア抗体価調査によると，2005年および2010年度の統計において，50歳代では著明にジフテリア抗体価が低下していることが分かる（図2）[10][11]．50歳代の感染症例が多いことに関しては，この抗体価の低下が感染，発症の原因と推察される．

本症例の感染経路についても，飼い猫の眼脂から *C. ulcerans* が検出されたため，この猫が感染源であったと考えられる．本邦の第1例目の報告においても多数の猫の飼育歴があり，感冒症状を呈した猫の出現後に患者は発症したと報告されている．国立感染症研究所のホームページ（http://www.nih.go.jp/niid/bac2/Coryne_ulcerans/）によると，2011年11月現在，本邦で発見，報告されている全8例中5例において猫の飼育歴がある

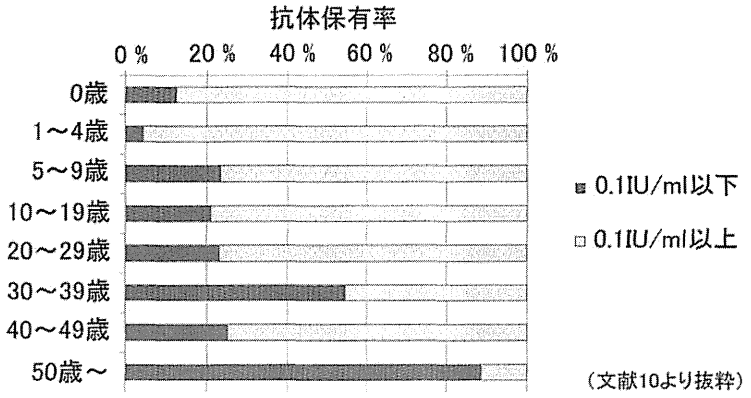

図2　年齢別ジフテリア抗体保有率（東京都）
0.1IU/ml 以上で感染防御レベルといわれているが，50歳以上では感染防御レベルに達しているのはごくわずか11％に過ぎない（文献10）より引用）．

表1　国内発症例のまとめ（国立感染症研究所の報告12）より引用，一部改編）

NO	発生年月日	年齢	発生場所	症状	その他	毒素原性
1	2001/2	52	千葉県	呼吸困難，咽頭痛，発熱，上咽頭と喉頭に偽膜	ネコ20匹飼育中（1匹死亡後発症）	＋
2	2002/10	54	千葉県	咽頭痛，発熱，上咽頭と右咽頭側索に偽膜	1例目と同地区に居住	＋
3	2005/9	58	岡山県	右頸部腫脹，軽度の咳	飼育犬が死亡後発症	＋
4	2005/10	51	大分県	肺に多発性空洞病変，咳，痰，発熱	ネコ12匹飼育	＋
5	2006/7	57	神奈川県	咽頭痛，上咽頭から中咽頭に偽膜，咳，発熱	インコ飼育	＋
6	2009/1	57	東京都	咽頭痛，中咽頭の偽膜，鼻空後方の粘液	飼育していた感冒症状を呈したネコから感染	＋
7	2010/9	51	茨城県	咽頭痛，上咽頭から中咽頭の白苔	飼い猫から感染	＋
8	2010/11	50	神奈川県	呼吸苦と左前胸部腫脹，腋下リンパ節腫脹	飼い猫から感染	＋

（表1）．また，本例を含めて3例で猫から *C. ulcerans* が検出されている．飼い犬よりも飼い猫からの感染が疑われる症例が多い理由としては，飼い猫は屋外において *C. ulcerans* を媒介する野良猫と接触を持つ機会が多いことが原因の一つと考えられる．本症例においても感染源となった飼い猫は屋外で野良猫との接点があった．厚生労働省研究班の報告によると，保健所に収容されたイヌやネコの咽頭ぬぐい液の検査で *C. ulcerans* の分離，もしくはジフテリア毒素遺伝子の検出が以下のように確認されている．大分県では9.8％（92例中9例），岡山県では5.9％（85例中5例），愛媛県では5.0％（101例中5例）が報告されており，およそ5-10％の野良猫，野良犬が *C. ulcerans* を保菌しているといえる[12][13][14]．これらの事実から，*C. ulcerans* は日本全土に広く存在しているものと考えられる．典型的な *C. diphtheria* はヒトのみを宿主とするため，ワクチンなどによる感染予防策が比較的容易である．しかし，*C. ulcerans* は，猫のような身近な動物を含めた多彩な動物を宿主とするため，その点で感染予防策がより困難である．予防法として，①動物からの感染予防としては風邪様症状（鼻水，くしゃ

み等）や皮膚炎の動物と接触した後は手と衣類の消毒を徹底する，②50歳以上の人はジフテリア抗体の抗体価が極めて低くなっている可能性があり，特に動物を飼育している場合はジフテリアワクチン追加接種を検討する，の二つが推奨される．

病歴や生活歴からなどから *C. ulcerans* 感染症を疑った場合，病変の培養検査を提出後，速やかにマクロライド系抗菌薬の治療を行い，症状と経過にあわせて必要な対処をとるのが望ましい．英国および米国CDC(Centers for Disease Control and Prevention）のガイドラインでは，抗毒素療法と抗菌薬（エリスロマイシンやペニシリンGを２週間投与）の併用が推奨されている．特に *C. ulcerans* 感染症が強く疑われ，症状が重篤な場合には，菌種同定検査結果を待たずに抗毒素療法（４万-10万単位）を併用する方法も推奨されている．抗毒素療法自体に静菌作用はないものの早期に抗毒素療法を行うことにより，気道からの *C. ulcerans* の除去の促進，毒素の新たな産生を抑制することが可能となり，その結果，患者を重篤な状態から救うことができるとされている[8]．しかしながら，本邦では治療ガイドラインがなく，また抗毒素血清が入手困難であり，今後検討すべき課題である．また *C. ulcerans* 感染症が確定した場合は，厚生労働省健康局への情報提供が求められている．

ま と め

飼い猫から感染した *C. ulcerans* のヒト感染例を経験した．耳鼻咽喉科領域に関連した部位の症状を来すことが多く，また，古典的ジフテリア症状を呈した場合に重症化することもある疾患であるため，迅速かつ適切な検査による確定診断が重要である．われわれ耳鼻咽喉科医は，急性咽頭炎の鑑別疾患としてこの疾患を念頭に置き，的確に診断する必要があると思われる．

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http ://idsc.tokyo-eiken.go.jp/epid/2006/tbkj2712.html
東京都微生物検査情報　2011 ; 32 : 3 .
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本稿を終えるにあたり，病理学的に *C. ulcerans* を同定してくださった，国立感染症研究所細菌第二部の小宮貴子先生，山本明彦先生，高橋元秀先生および，厚生労働科学研究費補助金（新型インフルエンザ等新興再興感染症研究事業）ワンヘルス理念に基づく動物由来感染症制御に関する研究班に深謝いたします．

なお，本論文の要旨は，2011/５/19の日本耳鼻咽喉科学会（京都）において報告した．

利益相反に該当する事項はない.

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(2011年7月8日受稿　2012年1月20日受理)

A Case of Acute Pharyngitis Caused by *Corynebacterium Ulcerans* in Ibaraki Prefecture

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We report on the case of a 51-year-old woman who presented with refractory pharyngitis caused by toxigenic *Corynebacterium ulcerans* (*C. ulcerans*). Thick pseudomembrane formations and yellowish pus were observed in her nasopharynx. Based on her clinical course and history of breeding cats, we considered *C. ulcerans* infection as the possible diagnosis. She was treated with macrolide administration and her symptoms immediately improved. *C. ulcerans* was identified in pus from the patient's pharynx as well as in discharge material from her cat's eyes, and *C. ulcerans* was thought to have caused her pharyngitis. *C. ulcerans* is one of the infecting bacteria which can cause a zoonotic infection. In Japan, some cases with *C. ulcerans* infection from cats have been reported. It is important that we should consider *C. ulcerans* infection as a differential diagnosis of refractory pharyngitis.

Keywords: Corynebacterium ulcerane, zoonotion, refractory pharyngitis

Nippon Jibiinkoka Gakkai Kaiho (Tokyo) 115: 682-686, 2012

ネコにおけるジフテリア毒素産生型 *Corynebacterium ulcerans* および *Corynebacterium* 属菌保有状況調査（2010-2011）第2報

木全　恵子　　嶋　智子　　金谷　潤一　　磯部　順子　　嶋　一世　　廣田　昌幸[1]
保田　信一[2]　　綿引　正則　　佐多　徹太郎

Isolation of Toxigenic *Corynebacterium ulcerans* and *Corynebacterium* spp. from Cats (2010-2011). Second Report

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目的： *Corynebacterium* 属菌は人獣の皮膚や口腔内の常在菌の1つである．このうちジフテリア毒素を産生する *Corynebacterium diphtheriae* による感染症，ジフテリアはその感染力と症状の重篤度から2類感染症に指定されている [1].

近年，この*C.diphtheriae*の近縁菌である*C.ulcerans*, *C.psudotuberculosiss*がジフテリア毒素（diphtheriae toxin 以後 DT）と類似したジフテリア様毒素（diphtheriae like toxin 以後 DLT）産生性となり，ヒトにジフテリア様症状を引き起こす事例が報告された．特にジフテリア様毒素産生型 *C.ulcerans*（以下 *C.ulcerans*$^{Tox+}$）によるヒト感染事例が欧米，日本で報告され[1-3]，厚生労働省は*C.ulcerans*$^{Tox+}$によるジフテリア様症状について周知と感染者に関する情報提供を医療機関をはじめとする関係機関に依頼している [4,5]．また，2011年1月にはジフテリア届出基準の改正を行い，ジフテリア様毒素産生性の *C.ulcerans*・*C. psudotuberculosiss* と *C. diphtheriae* との鑑別に留意することを求めているが，*C. ulcerans*・*C.psudotuberculosiss* による感染症は現在，感染症法に基づく届出対象に含まれていない [6].

日本では *C.ulcerans*$^{Tox+}$によるヒト感染事例は2001年から2011年までに散発的に発生した事例9件が報告されている．うち3件からは患者と接触のあったネコから *C.ulcerans*$^{Tox+}$が分離された[1,7-10]．これらの事例ではヒトーヒト感染は確認されず，動物ーヒト感染が疑われたが，上記9例中7例はネコやイヌとの接触歴が確認されており，愛玩動物からの感染の可能性が示唆されている．高橋らは動物の *C.ulcerans*$^{Tox+}$保有状況の調査を行い，国内における *C.ulcerans*$^{Tox+}$分布状況は明らかになりつつある [8]．また，海外では *C.ulcerans*$^{Tox+}$に感染したネコ等の愛玩動物からの感染のほか，畜産動物及び生乳からの感染事例が報告されており，動物を媒介とする感染症として注目されている [11-13].

我々は2009年に富山県内における *C.ulcerans*$^{Tox+}$および *Corynebacterium* 属菌の愛玩動物における保有状況を調査する目的で，行政機関に引き取られたネコを対象に *Corynebacterium* の保有状況を調査し，報告した [14].

引き続き2010・2011年について富山県内における *C.ulcerans*$^{Tox+}$の愛玩動物における保有状況の調査を行ったので報告する．これらの調査は平成22年度・23年度厚生労働科学研究費補助金 新型インフルエンザ等新興・再興感染症研究事業「ワンヘルス理念に基づく動物由来感染症制御に関する研究（研究代表者 国立感染症研究所 山田章雄）」における研究小班「コリネバクテリウムに関する研究：研究分担者 国立感染症研究所 高橋元秀（平成22年度）・山本明彦（平成23年度）」の一環として行われた．

材料と方法： 2010・2011年に採取された147検体について *C.ulcerans*$^{Tox+}$の分離を行った．その内訳は富山県動物管理センターに引き取られたネコの咽頭ぬぐいスワブ116検体，ネコ鼻汁スワブ6検体，開業獣医より採取されたネコ鼻汁スワブ23検体，イヌ鼻汁スワブ2検体である．

採取した咽頭ぬぐいスワブの分離培養は既報[14]に従い行った．勝川変法荒川培地（以下K培地，日研生物医学研究所）にスワブを塗抹し24・30・48・72時

1．富山県動物管理センター，2．ドリトル動物病院

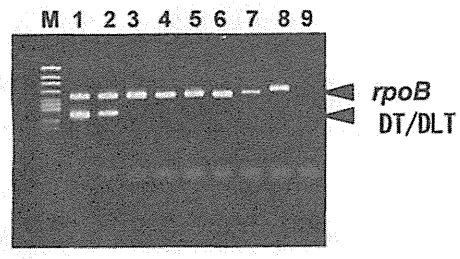
間後に生育したコロニーをヒツジ血液寒天培地（日水製薬）に採取した．K培地塗抹後のスワブをBacto™ Brain Heart Infusion (Becton, Dickinson and Campany) に振り出し，35℃で一晩培養した．

陽性対照株として国立感染症研究所より供与された *C.diphtheriae*$^{Tox+}$ PW8, *C.ulcerans*$^{Tox+}$ 旭0102を用いた．陰性対照株として *C.ulcerans*（国立感染症研究所より供与），*C.confusum* TC93, *C.ammoniagenes* TC300, *C.tuberculostrearicum* TC527, *C.auriscanis* TC522[14]を用いた．

C.ulcerans^{Tox+}の保有するDLT遺伝子のスクリーニングは以下の3つの方法で行った．DNA抽出は既報[14]に従い，①K培地上のコロニーの濃厚発育部分をかきとり鋳型DNAとしたSweep PCR，②ヒツジ血液寒天培地上で *C.ulcerans* を疑われる複数のコロニー（最大10コロニー）をひとつにまとめた混合鋳型DNAを用いたGroup PCR[15]，③増菌液抽出DNAのPCRを行った．DTおよびDLT遺伝子の検出は病原体検出マニュアル ジフテリア記載のDTプライマー[16]を用いた．

また，PCRによるスクリーニングの内部コントロールとしてRNAポリメラーゼβサブユニット遺伝子 *rpoB* を検出する *rpoB* プライマー [17]を用いた．これらの *rpoB* プライマーは,配列中に複数個所の重複塩基を保有しており（C2700F：CGWATGAACATYGGBCAGGT,C3130R: TCCATYTCRCCRAARCGCTG），*Corynebacterium* 属菌のほか他菌種の *rpoB* も増幅可能である（図1，レーン8，[17]）．上記検体の一部68検体について上記のDTプライマーと *rpoB* プライマーを混合した *rpoB*・DT dulplex PCR系を用い（図1），DTおよびDLT遺伝子を指標したスクリーニングを行った．*rpoB* プライマー C2700F，C3130R [17]を用い，*rpoB* 遺伝子の一部塩基配列434-452baseのPCR増幅が検出された *rpoB* 陽性・DT陰性の場合（図2A），DT陰性としてPCRによるスクリーニングは終了とした．*rpoB*・DT共に陰性の場合（図2B レーン20）はそれぞれ個別に *rpoB* とDTのPCRの再試を行った（図2C レーン25）．*rpoB*・DT共に陽性の場合（図2B レーン21）は，陽性となった混合鋳型DNAを構成するコロニーごとに鋳型DNAを抽出し，*rpoB*・DTのsingle PCRを行い，DT陽性株を特定した（図2C レーン26－34）．*rpoB*・DT dulplex PCR反応液は1×GoTaq® Hot Start Master Mix（Promega）に鋳型DNA2μl（20-50μg）とDTプライマー [16]と *rpoB* プライマー [17] 2組のプライマー各0.2μMを加え，95℃ 2分×1サイクル，（95℃ 20秒，55℃ 30秒，72℃ 1分）×35サイクル，72℃ 10分×1サイクルで行った．PCR後，3%アガロースゲル電気泳動とEtBr染色によりPCR産物を分離・検出した．

C.ulcerans 同定検査は病原体検出マニュアル ジフテリア[16]，既報[15]に従い，DLT遺伝子，*rpoB* 領域の塩基配列解析[14, 17]，DSS鑑別培地およびApi Coryne（ビオメリュー）にて同定した．毒素産生性・毒素抗体価測定，PFGEは国立感染症研究所にて実施した．



lane1 *C.diphtheriae* pw18 (Tox+)
lane2 *C.ulcerans* (Tox+)
lane3 *C.ulcerans* (Tox-)
lane4 *C.confusum* (Tox-)
lane5 *C.ammoniagenes* (Tox-)
lane6 *C.tuberculostearicum* (Tox-)
lane7 *C.auriscanis* (Tox-)
lane8 *E.coli*
lane9 H_2O
(M: ϕX174/*Hinc* II)

図1 ジフテリア毒素遺伝子・*rpoB*のduplex PCR

結果と考察： 2010-2011年に採取された147検体のうち *C.ulcerans*$^{Tox+}$ は1検体のみ検出され，残りの検体は全てDT陰性もしくはDLT陰性（*C.ulcerans*$^{Tox+}$ 陰性）であった．分離された株はDT PCR陽性でApiCoryneでは *C.ulcerans* ID99.7%であった．分離株のDT PCR産物と *rpoB* の塩基配列の一部（419bp）を解析したところ，DLT遺伝子および *C.ulcerans* type strain CIP106504の *rpoB* の塩基配列と100%一致した．また，ジフテリア毒素産生性試験により産生性が確認され，分離株は *C.ulcerans*$^{Tox+}$ 同定された．PFGEではこの分離株はヒト由来 *C.ulcerans*$^{Tox+}$ 岡山株[18]とパターンが一致した．

一方，*C.ulcerans*$^{Tox+}$ 陽性が分離されたネコは鼻汁症状であったが，血清の抗毒素価は検出限界以下であり，血中のジフテリア毒素は検出されなかった [19]．さらに同ネコの再検査では本菌は分離できなかった．これらのことから，今回 *C.ulcerans*$^{Tox+}$ 陽性となったネコの体内では *C.ulcerans*$^{Tox+}$ の感染が成立しておら

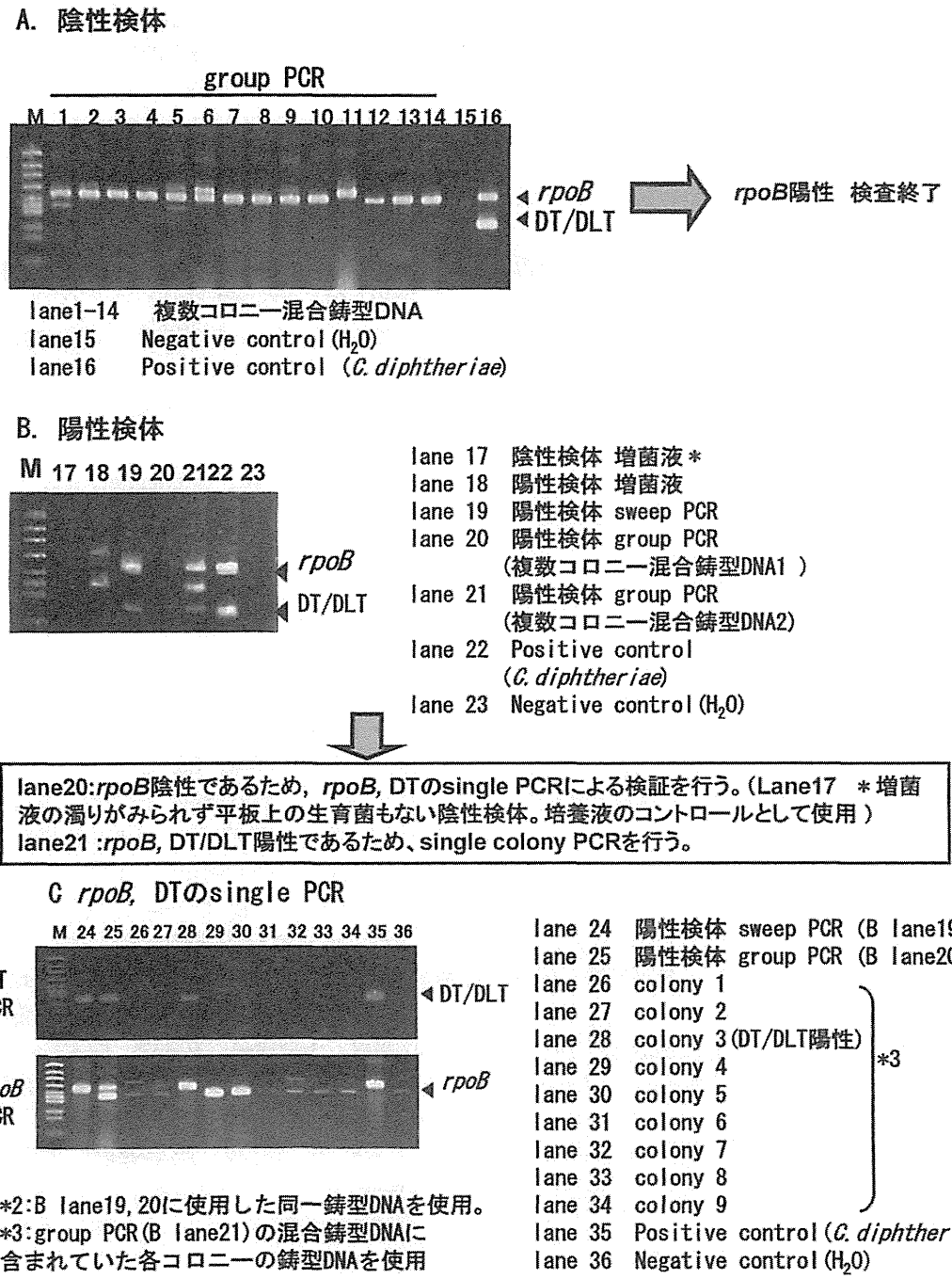


図2 ジフテリア毒素遺伝子・*rpoB*のduplex PCRによるスクリーニングの1例

ず，本菌は一時的な通過菌であり，鼻汁症状等の原因菌ではなかった可能性が考えられた．2009年調査分78検体を合わせ今回報告分の検体を合わせた225検体中本菌が検出された検体は1検体であり，全体の0.4%であった．以上から，本県においても環境中に *C.ulcerans*$^{Tox+}$ は存在していたが，ヒトへの感染を仲介するネコ等の感染の可能性は低いと考えられた．

今回，本調査の検体の一部68検体について *rpoB*・DT dulplex PCR を用いた．PCR でのスクリーニングには，鋳型 DNA 内の PCR 阻害物質による偽陰性の可能性を考慮に入れなければならない．今回 *Corynebacterium* 属菌の分離に特化した方法としてコリネ属菌の *rpoB* の一部を増幅するプライマーを利用した dulplex PCR を試行した．その結果，Group

PCR に用いた混合鋳型 DNA 90サンプルのうち21.1%が *rpoB*・DT dulplex PCR で *rpoB* 陰性・DT 陰性となり，*rpoB* PCR，DT PCR をそれぞれ再試した．dulplex PCR では *rpoB* が増幅しなかった鋳型 DNA を single PCR に用いた場合，PCR 増幅効率は低いが，これらの鋳型 DNA で *rpoB* の増幅が検出された。*rpoB* 陰性となった Group PCR の混合鋳型 DNA を構成する菌株22株と *rpoB* 陽性の混合鋳型 DNA を構成する菌株8株について16S r DNA による種同定を行ったところ，*Streptococcus sp*，*Leuconostoc mesenteroides*，*Enterococcus sp* が *rpoB* 陰性の混合鋳型 DNA に多く存在していた．これらの菌種と上記の dulplex PCR と single PCR における *rpoB* 増幅効率との関連性は不明である．以上の検討から DT PCR を *C.ulcerans*$^{Tox+}$ 分離のスクリーニングの主要な手段として用いる場合，PCR の内部コントロールなどの精度管理の構築が重要であると考えられた．

謝　辞

本稿を終えるにあたり，*C.ulcerans*$^{Tox+}$ の解析について多大なご協力を頂きました国立感染症研究所細菌第二部の山本明彦先生，小宮貴子先生，高橋元秀先生に感謝致します。

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J. Comp. Path. 2012, Vol. 146, 211–222

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EXPERIMENTALLY INDUCED DISEASE

Kinetics and Pathogenicity of Oral Infection by Equine Herpesvirus-9 in Mice and Suckling Hamsters

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Summary

The pathogenesis and kinetics of oral infection by equine herpesvirus (EHV)-9 were studied in mice and hamsters. After oral inoculation of 10^5 plaque-forming units (PFU) of virus, 1-week-old suckling hamsters showed varying severity of neurological disease from 72 hours post inoculation (hpi) and all of these animals had died by 96 hpi. Four-week-old ICR mice inoculated orally with 4×10^4 PFU of virus showed no clinical signs, but they developed erosive and ulcerative gastritis from 36 hpi. Varying degrees of encephalitis were seen in infected mice and hamsters, and the hamsters also developed myelitis by 96 hpi. Immunohistochemistry performed on whole body sections of suckling hamsters revealed the kinetics of spread of the virus to the central nervous system. EHV-9 antigen was detected initially in macrophages of the oral and lingual submucosa. At 36 hpi virus antigen was detected in the nerve fibres and pseudounipolar neurons of the trigeminal ganglion and at 96 hpi antigen was present in the myenteric plexuses of the intestine. Virus antigen was also detected in the liver, lungs and heart of affected animals. EHV-9 DNA was detected by polymerase chain reaction in the brain, blood and spinal cord of suckling hamsters at 36, 48 and 96 hpi. These findings show that EHV-9 may spread via the trigeminal nerve when mice and hamsters are inoculated orally with virus.

Keywords: equine herpesvirus-9; ICR mice; oral inoculation; suckling hamsters

Introduction

Equine herpesvirus (EHV)-9 is the newest member of the EHV family and was initially isolated from an outbreak of disease in Thomson's gazelles (*Gazella thomsoni*) that died from fulminant encephalitis (Fukushi *et al.*, 1997). Serologically, EHV-9 is most closely related to the recently emergent neurotropic pathogen EHV-1, but its DNA fingerprint differs from that of EHV-1 and other EHVs. The virus is estimated to have sequence homology of 95% to EHV-1 and EHV-8 and 60% to EHV-4 based on analysis of gene sequences encoding glycoprotein G and the conserved region of glycoprotein B (Fukushi *et al.*, 1997).

The infectivity of EHV-9 has been assessed in experimental infections by intranasal inoculation of mice and hamsters (Fukushi *et al.*, 2000), goats (Taniguchi *et al.*, 2000b), pigs (Narita *et al.*, 2000), horses (Taniguchi *et al.*, 2000a), dogs, cats (Yanai *et al.*, 2003a, b), common marmosets (Kodama *et al.*, 2007) and cattle (El-Habashi *et al.*, 2011). In those experiments, EHV-9 caused fatal encephalitis in all animal species except horses, which developed moderate encephalitis with less neuronal loss, moderate perivascular cuffing and gliosis. Recently, the virus was detected in a polar bear and a giraffe in an American

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0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2011.05.003

zoo (Schrenzel *et al.*, 2008; Donovan *et al.*, 2009). This wide host range of infection, as well as the ease of transmission by the nasal route, which is considered to be the natural route of infection (El-Habashi *et al.*, 2010a), has raised fears of emerging EHV-9 infection.

The pathogenesis of EHV-9-induced encephalitis following infection by the nasal route was studied using a suckling hamster model. EHV-9 propagated in the olfactory epithelium 12–24 hours post inoculation (hpi) and then gained access to the brain through the olfactory nerve (El-Habashi *et al.*, 2010a).

In previous studies we have reported the infectivity of EHV-9 by different routes of inoculation, including the ocular, oral and intraperitoneal routes (El-Habashi *et al.*, 2010b), but with no accurate description of the mechanism of induction of encephalitis. Therefore, further investigation is required in order to determine how EHV-9 reaches the brain following infection by routes other than nasal inoculation and to identify the mechanisms of EHV-9-induced encephalitis following infection by these alternative routes of administration. To that end, we are currently conducting investigations of intraperitoneal (El-Nahass *et al.*, 2011) and ocular inoculation of the virus (unpublished data).

The primary site of propagation of EHV-9 following oral inoculation has not yet been identified, nor is it known how the virus gains access to the brain after this infection or how long this process takes. The pathogenesis of infection by other neurotropic viruses (e.g. EHV-1, Hasebe *et al.*, 2002; Theiler's virus and poliovirus, Villarreal *et al.*, 2006) has been reported.

The aim of the present study was to determine the pathogenesis and kinetics of infection by EHV-9 following oral inoculation of mice and suckling hamsters.

Materials and Methods

Virus Culture

Madine-Derby bovine kidney (MDBK) cells were used for the propagation of EHV-9. The inocula were prepared by culturing the virus from the original seed stocks of EHV-9 (P19, fifth passage in MDBK cells) in MDBK cells. The virus was titrated by plaque formation assay on MDBK cells.

Animals and Virus Inoculation

Animals were provided with a basal pellet diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and bottled water *ad libitum*. The experiment was conducted in accordance with pertinent laws and related standard operating procedures on the treatment and use of laboratory animals. The experimental protocol was approved by the Animal Experiment Committee of the Faculty of Applied Biological Science at Gifu University, Japan.

ICR Mice. Forty-four 4-week-old male ICR mice were purchased from a breeder (SLC Inc., Hamamatsu, Japan). The mice were housed in plastic cages and kept in an isolated biohazard cabinet for approximately 1 week of acclimatization. The mice were divided into eight groups for virus inoculation ($n = 5$) and one control group ($n = 4$). Each group was inoculated orally with a single dose of 50 µl of 2×10^6 virus solution (10^5 plaque-forming units [PFU]). Mice were killed at 12, 24, 36, 48, 72, 96, 120 and 144 hpi. Control mice were inoculated orally with minimum essential medium (MEM). Animals were checked for clinical signs at least three times daily.

Syrian Hamsters. Ten 10-week-old female Syrian hamsters at 10 days of gestation were purchased from a breeder (SLC Inc.). The animals were housed in plastic cages and left to give birth. After birth, forty-five 7-day-old suckling hamsters were inoculated orally with a single dose of 20 µl of 2×10^6 of EHV-9 virus solution (4×10^4 PFU). Five animals were killed at 12, 24, 36, 48, 72, 96, 120 and 144 hpi. Four of these five animals were used for histopathology and immunohistochemistry (IHC) and one provided samples for polymerase chain reaction (PCR). Animals were checked for clinical signs at least three times daily.

Collection and Processing of Tissues

The brain, different levels of the spinal cord, tongue, stomach, small and large intestine, liver, spleen, heart and lungs were collected from young adult ICR mice and fixed in 10% neutral buffered formalin. To examine the entire length of the digestive tract, a 'Swiss roll' technique was applied (Fig. 1) (Moolenbeek and Ruitenberg, 1981).

To examine the whole body in section for detection of virus antigen, the suckling hamsters were bisected sagittally and fixed in 10% neutral buffered formalin. Suckling hamsters prepared in this fashion did not require decalcification before sectioning (El-Habashi *et al.*, 2010a).

Immunohistochemistry

For IHC, sections from paraffin wax-embedded tissues of mice and hamsters were immunolabelled with rabbit antiserum specific for EHV-9 (Veterinary Microbiology Laboratory, Gifu University; 1 in 800 dilution) by the avidin–biotin complex (ABC) immunoperoxidase