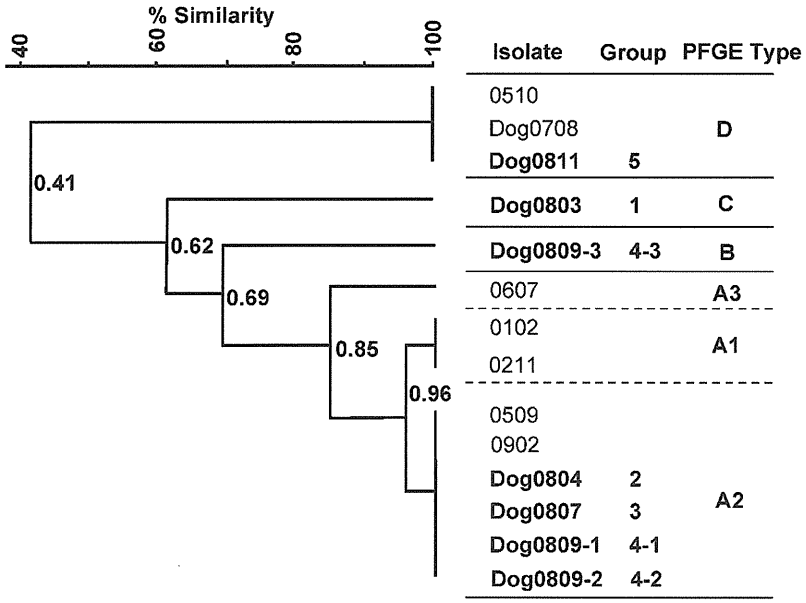

Fig. 2. Phylogenetic analysis of *C. ulcerans* isolates. *C. ulcerans* dog isolates obtained in this study (indicated in bold) as well as Japanese human isolates reported previously (Asakura *et al.*, 2006; Hagiwara *et al.*, 2006; Hatanaka *et al.*, 2003; Komiya *et al.*, 2010; Noguchi *et al.*, 2009; 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 $\geqslant 2$ μg ml^{-1} in all strains, judged as resistant or intermediate. In addition, the LVFX MIC was 2 μg ml^{-1} in six isolates and $\leqslant 0.25$ μg 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

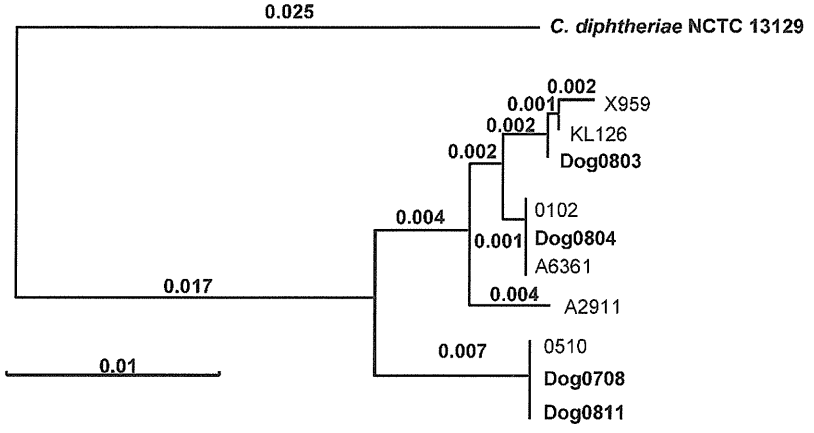

Fig. 3. Phylogenetic analysis based on nucleotide sequences of the *tox* genes. The *tox* gene sequences for the *C. ulcerans* isolates obtained in the present study were determined and compared to the previously published *tox* gene sequences of *C. ulcerans* strains isolated from humans and the *C. 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

tox type or strain	*Corynebacterium ulcerans*						*C. diphtheriae*
	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 μg 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 toxaemic 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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REFERENCES

Asakura, S., Katayama, N., Hara, Y., Sunami, K., Ohgami, T., Takeda, Y., Mimura, S., Yamatori, I., Ohkusu, K. & other authors (2006). A case of cervico-lymphadenitis due to *Corynebacterium ulcerans*, 2005 – Okayama. *Infect Agents Surveill Rep* **27**, 124–125 (in Japanese).

Bonnet, J. M. & Begg, N. T. (1999). Control of diphtheria: guidance for consultants in communicable disease control. World Health Organization. *Commun Dis Public Health* **2**, 242–249.

Bostock, A. D., Gilbert, F. R., Lewis, D. & Smith, D. C. M. (1984). *Corynebacterium ulcerans* infection associated with untreated milk. *J Infect* **9**, 286–288.

De Zoysa, A., Efstratiou, A., George, R. C., Jahkola, M., Vuopio-Varkila, J., Deshevoi, S., Tseneva, G. Y. & Rikushin, Y. (1995). Molecular epidemiology of *Corynebacterium diphtheriae* from north-western Russia and surrounding countries studied by using ribotyping and pulsed-field gel electrophoresis. *J Clin Microbiol* **33**, 1080–1083.

De Zoysa, A., Hawkey, P. M., Engler, K., George, R., Mann, G., Reilly, W., Taylor, D. & Efstratiou, A. (2005). Characterization of toxigenic *Corynebacterium ulcerans* strains isolated from humans and domestic cats in the United Kingdom. *J Clin Microbiol* **43**, 4377–4381.

Dias, A. A. S. O., Silva, F. C., Jr, Pereira, G. A., Souza, M. C., Camello, T. C. F., Damasceno, J. A. L. D., Pacheco, L. G. C., Miyoshi, A., Azevedo, V. A. & other authors (2010). *Corynebacterium ulcerans* isolated from an asymptomatic dog kept in an animal shelter in the metropolitan area of Rio de Janeiro, Brazil. *Vector Borne Zoonotic Dis* **10**, 743–748.

Efstratiou, A. & George, R. C. (1999). Laboratory guidelines for the diagnosis of infections caused by *Corynebacterium diphtheriae* and *C. ulcerans*. World Health Organization. *Commun Dis Public Health* **2**, 250–257.

Engler, K. H., Glushkevich, T., Mazurova, I. K., George, R. C. & Efstratiou, A. (1997). A modified Elek test for detection of toxigenic corynebacteria in the diagnostic laboratory. *J Clin Microbiol* **35**, 495–498.

Hagiwara, N., Horike, S., Kasahara, S., Suganuma, T., Aizawa, T., Nakamura, K., Nakanishi, M., Yatsuhashi, R., Okazaki, N. & other authors (2006). A diphtheria-like case due to *Corynebacterium ulcerans*, July 2006 – Kanagawa. *Infect Agents Surveill Rep* **27**, 334–335 (in Japanese).

Hatanaka, A., Tsunoda, A., Okamoto, M., Ooe, K., Nakamura, A., Miyakoshi, M., Komiya, T. & Takahashi, M. (2003). *Corynebacterium ulcerans* diphtheria in Japan. *Emerg Infect Dis* **9**, 752–753.

Hatanaka, A., Kamata, T., Tazaki, A., Honda, K., Yamamoto, A., Komiya, T. & Takahashi, M. (2011). A case of *Corynebacterium ulcerans* diphtheria – Ibaraki. *Infect Agents Surveill Rep* **32**, 19–20 (in Japanese).

Hogg, R. A., Wessels, J., Hart, J., Efstratiou, A., De Zoysa, A., Mann, G., Allen, T. & Pritchard, G. C. (2009). Possible zoonotic transmission of toxigenic *Corynebacterium ulcerans* from companion animals in a human case of fatal diphtheria. *Vet Rec* **165**, 691–692.

Jacherts, D. (1956). [The DSS agar, a polyvalent culture medium for the differentiation of diphtherial and pseudodiphtherial bacteria]. *Zentralbl Bakteriol* [*Orig*] **167**, 306–309 (in German).

Katsukawa, C., Kawahara, R., Inoue, K., Ishii, A., Yamagishi, H., Kida, K., Nishino, S., Nagahama, S., Komiya, T. & other authors (2009). Toxigenic *Corynebacterium ulcerans* isolated from the domestic dog for the first time in Japan. *Jpn J Infect Dis* **62**, 171–172.

Khamis, A., Raoult, D. & La Scola, B. (2004). *rpoB* gene sequencing for identification of *Corynebacterium* species. *J Clin Microbiol* **42**, 3925–3931.

Komiya, T., Seto, Y., De Zoysa, A., Iwaki, M., Hatanaka, A., Tsunoda, A., Arakawa, Y., Kozaki, S. & Takahashi, M. (2010). Two Japanese *Corynebacterium ulcerans* isolates from the same hospital: ribotype, toxigenicity and serum antitoxin titre. *J Med Microbiol* **59**, 1497–1504.

Lartigue, M.-F., Monnet, X., Le Flèche, A., Grimont, P. A. D., Benet, J.-J., Durrbach, A., Fabre, M. & Nordmann, P. (2005). *Corynebacterium ulcerans* in an immunocompromised patient with diphtheria and her dog. *J Clin Microbiol* **43**, 999–1001.

Miyamura, K., Tajiri, E., Ito, A., Murata, R. & Kono, R. (1974). Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. II. Comparison with the rabbit skin method and practical application for seroepidemiological studies. *J Biol Stand* **2**, 203–209.

Nakao, H. & Popovic, T. (1997). Development of a direct PCR assay for detection of the diphtheria toxin gene. *J Clin Microbiol* **35**, 1651–1655.

Nakao, H., Pruckler, J. M., Mazurova, I. K., Narvskaia, O. V., Glushkevich, T., Marijevski, V. F., Kravetz, A. N., Fields, B. S., Wachsmuth, I. K. & Popovic, T. (1996). Heterogeneity of diphtheria toxin gene, *tox*, and its regulatory element, *dtxR*, in *Corynebacterium diphtheriae* strains causing epidemic diphtheria in Russia and Ukraine. *J Clin Microbiol* **34**, 1711–1716.

Noguchi, Y., Tsunoda, A., Ken, K., Komiya, T., Yamamoto, A. & Takahashi, M. (2009). A case of acute pharyngitis caused by *Corynebacterium ulcerans*, January 2009 – Tokyo. *Infect Agents Surveill Rep* **30**, 188–189 (in Japanese).

Nureki, S.-i., Miyazaki, E., Matsuno, O., Takenaka, R., Ando, M., Kumamoto, T., Nakano, T., Ohkusu, K. & Ezaki, T. (2007). *Corynebacterium ulcerans* infection of the lung mimicking the histology of Churg-Strauss syndrome. *Chest* **131**, 1237–1239.

Reinhardt, D. J., Lee, A. & Popovic, T. (1998). Antitoxin-in-membrane and antitoxin-in-well assays for detection of toxigenic *Corynebacterium diphtheriae*. *J Clin Microbiol* **36**, 207–210.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schuhegger, R., Schoerner, C., Dlugaiczyk, J., Lichtenfeld, I., Trouillier, A., Zeller-Peronnet, V., Busch, U., Berger, A., Kugler, R. & other authors (2009). Pigs as source for toxigenic *Corynebacterium ulcerans*. *Emerg Infect Dis* **15**, 1314–1315.

Seto, Y., Komiya, T., Iwaki, M., Kohda, T., Mukamoto, M., Takahashi, M. & Kozaki, S. (2008). Properties of corynephage attachment site and molecular epidemiology of *Corynebacterium ulcerans* isolated from humans and animals in Japan. *Jpn J Infect Dis* **61**, 116–122.

Sing, A., Hogardt, M., Bierschenk, S. & Heesemann, J. (2003). Detection of differences in the nucleotide and amino acid sequences of diphtheria toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* causing extrapharyngeal infections. *J Clin Microbiol* **41**, 4848–4851.

Sing, A., Bierschenk, S. & Heesemann, J. (2005). Classical diphtheria caused by *Corynebacterium ulcerans* in Germany: amino acid sequence differences between diphtheria toxins from *Corynebacterium diphtheriae* and *C. ulcerans*. *Clin Infect Dis* **40**, 325–326.

Sykes, J. E., Mapes, S. L., Lindsay, L. L., Samitz, E. & Byrne, B. A. (2010). *Corynebacterium ulcerans* bronchopneumonia in a dog. *J Vet Intern Med* **24**, 973–976.

Takahashi, M. (2009). *Risk Assessment of Zoonotic Diseases by Ecological Approach*, H19-Shinkou-ippan-009. Tokyo: Ministry of Health, Labour and Welfare.

Tinsdale, G. F. W. (1947). A new medium for the isolation and identification of *C. diphtheriae* based on the production of hydrogen sulphide. *J Pathol Bacteriol* **59**, 461–466.

Tiwari, T. S., Golaz, A., Yu, D. T., Ehresmann, K. R., Jones, T. F., Hill, H. E., Cassiday, P. K., Pawloski, L. C., Moran, J. S. & other authors (2008). Investigations of 2 cases of diphtheria-like illness due to toxigenic *Corynebacterium ulcerans*. *Clin Infect Dis* **46**, 395–401.

Wellinghausen, N., Sing, A., Kern, W. V., Perner, S., Marre, R. & Rentschler, J. (2002). A fatal case of necrotizing sinusitis due to toxigenic *Corynebacterium ulcerans*. *Int J Med Microbiol* **292**, 59–63.

Yoshimura, Y., Yamamoto, A. & Komiya, T. (2010). A case of axillary lymph node abscess caused by percutaneous infection of *Corynebacterium ulcerans* through scratch by a pus-discharging cat, June 2010. *Infect Agents Surveill Rep* **31**, 331 (in Japanese).

ORIGINAL ARTICLE

Seroprevalence of Tularemia in Wild Bears and Hares in Japan

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Impacts

- Serological assays for tularemia were performed with 431 Japanese black bears and 293 Japanese hares samples.
- All eight seropositive samples were originated from Japanese black bears from the Tohoku district, northeastern region of the Honshu, Japan.
- Japanese black bears can be used as a sentinel for tularemia.

Keywords:
Francisella tularensis; tularemia; wild animals; seroprevalence

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Summary

Tularemia is a zoonotic disease caused by *Francisella tularensis*. The distribution of the pathogen in Japan has not been studied well. In this study, seroprevalence of tularemia among wild black bears and hares in Japan was determined. Blood samples collected from 431 Japanese black bears (*Ursus thibetanus japonicus*) and 293 Japanese hares (*Lepus brachurus*) between 1998 and 2009 were examined for antibodies against *F. tularensis* by micro-agglutination test (MA) or enzyme-linked immunosorbent assay. By subsequent confirmatory tests using western blot (WB) and indirect immunofluorescence assay (IFA), eight sera from Japanese black bears were definitely shown to be seropositive. All of these eight bears were residents of the northeastern part of main-island of Japan, where human tularemia had been reported. On the other hand, no seropositive Japanese hares were found. These results suggest that Japanese black bears can serve as sentinel for tularemia surveillance and may help understand the distribution of *F. tularensis* throughout the country. This is the first report on detection of antibody to *F. tularensis* in black bears of Japan.

Introduction

Tularemia is a zoonotic disease caused by *Francisella tularensis*, highly infective, intracellular gram-negative coccobacilli. It is primarily a disease of wild animals: mainly lagomorphs and rodents. The disease occurs throughout the northern hemisphere including North America, Russia, Europe and Japan. In North America and Europe, 100–200 human tularemia cases are reported every year (Ellis et al., 2002). Humans are infected through contact with infected animals, arthropod bites, ingestion of contaminated water or food, and inhalation of infective aerosols (Ellis et al., 2002). The clinical type and severity of the disease is dependent on the route of infection. Predominant symptoms are high fever, enlarged lymph nodes, and ulcer at the site of bacterial entry (Ellis et al., 2002). In animals, the severity of the disease varies among species. In susceptible animals such as mice severe collapses are followed by a fatal septicaemia. Other animal species such as cats, dogs and cattle are relatively resistant to the infection (Hopla, 1974).

Understanding of the distribution of the pathogen in animal populations is of particular importance when studying zoonoses. The seroprevalence of *F. tularensis* in wild animals in North America and Europe has been reported for bears (Binninger et al., 1980; Chomel et al., 1998), hares (Mörner et al., 1988; Frölich et al., 2003), rabbits (Shoemaker et al., 1997; Berrada et al., 2006) and wild boars (Al Dahouk et al., 2005). These data are indispensable to assess the risk of future occurrence of tularemia in humans and domestic animals as well as to identify the natural reservoir of *F. tularensis*.

In Japan, tularemia was first reported in 1924, and approximately 1400 human cases have been reported since then (Ohara et al., 1991). The annual incidence of tularemia has decreased from the middle of the 1960s and it became extremely rare thereafter (Ohara et al., 1996).

Most of human cases occurred in the Tohoku district, the northeastern part of the largest island, Honshu, Japan. The pathogens had been isolated from humans, hares, ticks, and shrew-mole, and a number of wild animals (such as hare, bear, or squirrel) have been suggested to have epidemiological links to human infections (Ohara et al., 1996). However, epidemiological study on wild animals is scarce and the distribution of *F. tularensis* in environment is not well understood.

We developed several tools for diagnosis of tularemia, such as monoclonal antibodies (Hotta et al., 2007), and protocols for DNA amplification and detection (Fujita et al., 2006; Uda et al., 2007). We also reported molecular epidemiological characteristics of Japanese *F. tularensis* isolates (Fujita et al., 2008). In this study, to assess the potential risk of occurrence of tularemia by understanding the distribution of *F. tularensis* in wild animals, we investigated whether Japanese hares and black bears have specific antibodies against *F. tularensis*.

Materials and Methods

Blood samples

Sera or plasma from 431 wild Japanese black bears were collected from 11 prefectures, Iwate, Fukushima, Ibaraki, Yamanashi, Nagano, Gifu, Shiga, Kyoto, Hyogo, Tottori and Tokyo between 1998 and 2007 (Fig. 1).

Samples from 293 wild Japanese hares were collected from nine prefectures, Aomori, Iwate, Akita, Yamagata, Fukushima, Niigata, Kochi, Miyazaki and Kagoshima during the winters (November–April) from 2005 to 2009 (Fig. 1). Apparently healthy wild Japanese hares were captured by licensed hunters. The blood samples were collected onto filter papers (Toyo-Roshi Ltd, Tokyo, Japan) or into plastic tubes. The filter papers were incubated with 1 ml of phosphate-buffered saline (PBS), pH 7.2, containing 0.5% (vol/vol) Tween 20 at 4°C for 4 h on a rotator. After centrifugation at 13 000 ***g*** for 3 min, the supernatant was collected and stored at −80°C until use. Because the filter paper was designed to retain 250 μl of whole blood, resulting extracts were regarded as a 1 : 50 dilution of the sera (De Swart et al., 2001). Rabbit defibrinated blood (800 μl; Nippon Biotest Laboratories Ltd, Tokyo, Japan) mixed with the sera from *F. tularensis* immunized and normal rabbits (200 μl) were used as positive and negative control, respectively. The blood samples collected to tubes were ordinarily processed to obtain sera.

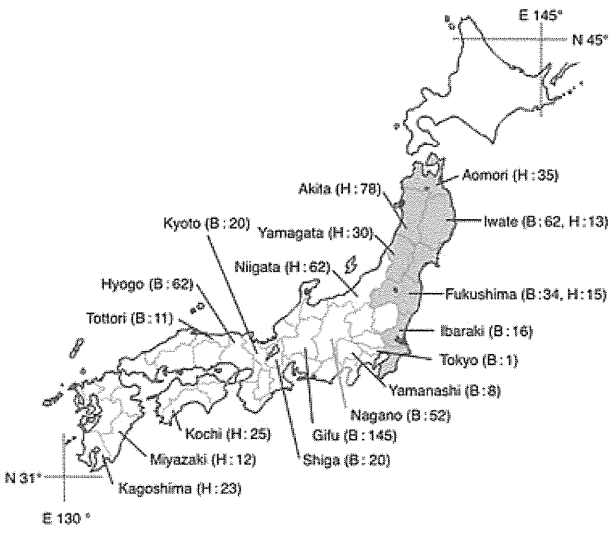

Fig. 1. Map of Japan showing the areas where samples were collected. The numbers of samples collected in each area was shown in parentheses (B, Japanese black bears; H, Japanese hares). The areas coloured grey are the prefectures where more than 50 cases of human tularemia have been reported previously (Ohara et al., 1996).

Bacterial antigens

Francisella tularensis (Yama strain), *Francisella novicida* (U112 strain), and *Francisella philomiragia* (029 strain) were kindly provided by Dr Hiromi Fujita, Ohara Research Laboratory, Fukushima, Japan. *Francisella tularensis* were propagated on Difco™ Eugon agar (Becton, Dickinson and Company, Sparks, MD, USA) with chocolatized 8% (vol/vol) sheep blood under the biosafety level 3 condition. *Francisella tularensis* LPS was purified using a LPS Extraction kit (iNtRON Biotechnology, Kyungki-Do, Korea) according to the protocol provided by the supplier. *Brucella abortus*, *Brucella canis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pasteurella aerogenes* were propagated in our laboratory as described previously (Hotta et al., 2007).

Micro-agglutination test (MA)

Sera or plasma samples from Japanese black bears and Japanese hares were screened by MA according to Sato et al. (1990). Twenty-five microlitres of 2-fold serial dilution of samples were mixed with an equal volume of antigen solution in wells of a round type micro-titre plate. Judgment was made after incubation at 37°C for 18 h. The agglutination titre was expressed as the reciprocal of the highest serum dilution showing a positive response to the antigens.

Enzyme-linked immunosorbent assay (ELISA)

The extracts from the filter paper were screened by ELISA. Six micrograms of purified *F. tularensis* LPS was dispensed into wells of a flat type 96-well microtitre plate and the plate was incubated at 4°C overnight. After washing five times with PBS containing 0.1% (vol/vol) of Tween 20

(PBST), the wells were incubated with PBST containing 3% (wt/vol) non-fat milk at RT for 1 h. After further washing with PBST, samples were added to the wells at a final dilution of 1 : 100 and the plate was incubated at 37°C for 1 h (Shoemaker et al., 1997). The plate was further incubated with 1 : 8000 horseradish peroxidase (HRP) conjugated anti-rabbit IgG (ICN Products; ICN Pharmaceuticals, Inc., Aurora, OH, USA) at 37°C for 1 h. The bound conjugate was colour developed by addition of 100 μl of substrate solution (0.003% H_2O_2, 0.05 M citric acid and 1 mg/ml of 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid). Absorbance at 405 nm was read by the ELISA reader model 680XR (BioRad, Hercules, CA, USA). Sera from immune and normal rabbits were used as positive and negative control, respectively. All samples were tested in duplicate and the samples that showed OD value over the cut-off value (mean + 2SD) were considered as positive (Al Dahouk et al., 2005).

SDS-PAGE and western blotting (WB)

Whole cell lysate and purified LPS of *F. tularensis* Yama strain were subjected to SDS-PAGE using 12.5% gel and antigens were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corporation, Bedford, MA, USA). After incubating in Immunoblock (Dainippon Sumitomo Pharma, Tokyo, Japan) at RT for 1 h followed by several washings with PBST, the PVDF membrane was incubated with the samples appropriately diluted with a 4-fold dilution of Immunoblock (Dainippon Sumitomo Pharma) at RT for 1 h. Dilution of Japanese black bear and Japanese hare samples were 1 : 1000 or 1 : 200 times, respectively. After further washings with PBST three times, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (ICN Products; ICN Pharmaceuticals, Inc.) or HRP-conjugated recombinant protein A (Pierce, Rockford, IL, USA) at a dilution of 1 : 8000 at RT for 1 h. Finally, antigen reacted with the samples were visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.02% 3,3′-diaminobenzidine (Wako Pure Chemicals, Osaka, Japan) and 0.003% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). Samples were considered to contain specific antibodies when the typical LPS ladder-banding pattern was recognized (Al Dahouk et al., 2005) regardless of whether there were high background reaction. Mouse monoclonal antibody against LPS and serum from mouse experimentally infected with *F. tularensis* were used as positive control.

Indirect immunofluorescence assay

The whole bacterial cells of *F. tularensis* Yama strain suspended in 10 μl saline were placed onto each well of the 24 spots slides (Matsunami Glass Ind., Ltd, Osaka, Japan), air-dried, and fixed with pure methanol at RT for 15 min. Twenty to 160-fold dilution of samples were added to the slides and incubated at 37°C for 30 min. After washings with PBS and distilled water, the slides were incubated with 10 μl of protein A conjugated with fluorescent isothiocyanate (Zymed Laboratories, Invitrogen Immunodetection, Carlsbad, CA, USA) at a dilution of 1 : 200 with PBS at 37°C for 45 min. The specific fluorescence was observed under a Olympus BX51 UV microscope (Olympus, Tokyo, Japan). Because a number of non-specific reactions were observed at dilution 1 : 20, samples were considered positive when they reacted with the antigens at dilutions of greater than 1 : 40.

Criterion of positive reaction

When samples which tested positive in MA or ELISA gave rise to positive reactions both in WB and immunofluorescence assay (IFA), we considered that these samples contained specific antibody directed to *F. tularensis*.

Cross-reactivity with other bacterial antigen

The samples reacted with *F. tularensis* in both WB and IFA were further tested for their reactivity to other bacterial antigens including *F. novicida*, *F. philomiragia*, *B. abortus*, *B. canis*, *E. coli*, *K. pneumoniae* and *P. aerogenes* by ELISA.

Results

Screening assays

At first, 431 sera or plasma from Japanese black bears and 47 sera from Japanese hares were screened for the antibodies to *F. tularensis* using the MA test. Sixteen samples obtained from black bears of Iwate and seven from Fukushima prefectures agglutinated the antigen with titres from 10 to 80 (Table 1). No sample originated from other areas showed agglutination at all. Forty-seven sera from hares did not show any agglutination (data not shown). Because of limited amount of samples, all blood samples of hares extracted from the filter papers were tested by ELISA. Out of 293 samples, only one sample of a hare captured in Akita showed high OD value (1.47).

Confirmatory assays

Twenty-four samples (23 bears and one hare) tested positive in screening assays were subjected to WB and IFA together with several negative samples in screening assays to make sure that these samples did contain specific antibodies directed to *F. tularensis*. Ten samples from

Table 1. Antibody prevalence to *Francisella tularensis* in Japanese black bears

Area	No. sample	No. positive			No. positive in all assays	Positive rate (%)
		Screening	Confirmatory assay*			
		MA	WB	IFA		
Iwate	62	16	10	8	8	12.9
Fukushima	34	7	0	0	0	
Gifu, Hyogo, Kyoto, Nagano, Shiga, Ibaraki, Tottori, Yamanashi, Tokyo	335	0	NT	NT	0	
Total	431	23	10	8	8	1.9

MA: agglutination at dilutions of 1 : 10 or higher were considered to be positive.
WB: LPS banding pattern observed with 1000 time dilution were considered to be positive.
IFA: immunofluorescence assay titre at dilutions of 1 : 40 or higher were considered to be positive.
NT, not tested.
*Confirmatory assays were performed only for MA positive samples.

Japanese black bears reacted with both whole cell lysate and LPS antigens with similar banding pattern (Fig. 2, sheet nos. 1–10). In IFA, eight of these 10 samples reacted with whole cell antigen at 1 : 40 or 1 : 80 (summarized in Table 1). These eight positive samples did not react with antigens prepared from bacterial species other than *F. tularensis* in ELISA (data not shown). Fifteen remaining samples of screening positive did not give rise to positive reaction against *F. tularensis* in IFA at 1 : 40. According to the criterion described in the *Materials and Methods*, eight samples, which were positive in both WB and IFA, were considered to contain specific antibody to *F. tularensis*. On the other hand, ELISA-positive sample from a Japanese hare did not react with purified LPS in WB (Fig. 2, lane 12). There were, however, several bands when whole cell lysate was used as antigen. Because similar bands were also found when ELISA-negative samples from hares were subjected to WB, the presence of those bands was probably due to non-specific reaction. In addition, this sample did not show any positive reaction in IFA at 1 : 20. We therefore concluded that samples from hares were negative.

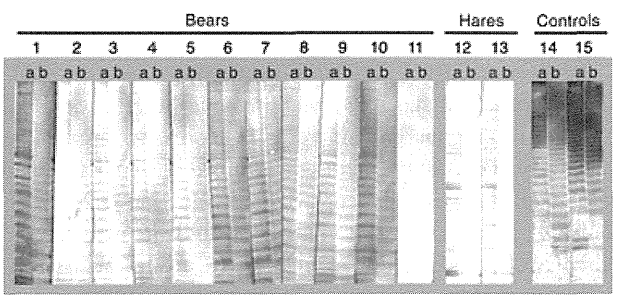

Fig. 2. Detection of antibodies to *Francisella tularensis* antigen by western blot. Sera from 10 black bears (sheets 1–10) reacted specifically with whole cell lysate (a) and purified LPS (b). Thirteen other samples tested positive in the screening MA did not show any positive reaction with either antigens as represented by the sheet 11. Reactions of the screening positive and negative hares samples (sheets 12 and 13, respectively) did not react with LPS. Anti-*F. tularensis* LPS monoclonal antibody (sheet 14) and serum from mouse experimentally infected with *F. tularensis* (sheet 15) were used as positive controls.

Eight samples shown to be positive in all three assays (MA, WB and IFA) were obtained from the bears captured in Iwate between 1999 and 2003. Seven of them were from male bears older than 3 years of age (Table 2). No specific reaction was observed when samples from female bears and bears aged <2 years were examined. The overall prevalence of anti-*F. tularensis* antibody among bears in Japan was 1.9% (8/431) (Table 1); however, nearly one in 10 bears of Iwate had experiences of infections with *F. tularensis* (Table 2).

Discussion

Upon conducting serological survey in wild animals, confirmation of the specificity is extremely important, because false positive reactions are fairly common when samples taken from wild or feral animals are examined. Although MA and ELISA are commonly used for testing a large number of samples, the false-positive reactions may sometimes occur (Grebenchtchikov et al., 2002; CDC, 2008). Recently, WB and IFA have become confirmatory tests for detection of antibody to *F. tularensis* (Porsch-Ozcurumez et al., 2004; Magnarelli et al., 2007). To avoid inclusion of false positives, only samples tested positive in all three assays (MA or ELISA, WB and IFA) were regarded as definitely positive in this study. Because

Table 2. Distribution of age and gender of positive samples from Japanese black bears captured in Iwate

	Male		Female		Unknown		Total	
Age	Positive samples/*n*	%	Positive samples/*n*	%	Positive samples/*n*	%	Positive samples/*n*	%
<3	0/1		0/1				0/2	
3–5	3/15	20.0	0/10				3/25	12.0
6–9	2/11	18.2	0/5				2/16	12.5
>9	1/6	16.7	0/3				1/9	11.1
Unknown	1/5	20.0	0/1		1/4	25.0	2/10	20.0
Total	7/38	18.4	0/20		1/4	25.0	8/62	12.9

The blanks indicate no sample or 0%.

the criteria adopted in this study is rather strict, it seems possible that the samples regarded as negative here actually contain specific antibody to *F. tularensis*. Nevertheless we believe that the bears identified as seropositive in this assay had definitely been infected with *F. tularensis*. The lack of reaction with antigens prepared from irrelevant bacteria guaranteed the specificity of these tests (data not shown). This is the first report describing the detection of antibodies against *F. tularensis* in bears of Japan. These bears may be infected by direct contact with infected carcasses of hare or mice, or indirectly by bite of infected ticks. It is not known whether infected bears show clinical signs or symptoms, but it is generally thought that bears are relatively resistant (Hopla, 1974). Because Japanese black bears have been suspected as the source of human infection with *F. tularensis* (Ohara et al., 1996), investigating how bears got infected may help understand the ecology of zoonotic *F. tularensis* and the possible roles of bears in the maintenance of *F. tularensis* in nature.

Iwate prefecture is the only place where infected Japanese black bears are recognized. Out of eight samples, two were taken from the bears captured in 2003 (data not shown), indicating that *F. tularensis* still exists in Iwate. We could not definitively conclude that several bears originated from other areas had been infected with the bacteria, because one of the confirmatory tests gave negative results. Although Fukushima is endemic area of tularemia (Table 1), none of animals were proved to be seropositive to *F. tularensis*. This finding may be explained by the fact that the number of Japanese black bears is scarce in the Abukuma mountains where tularemia is most endemic in Fukushima (Francis and Moore, 1926; Yamazaki and Inaba, 2009). The prevalence of tularemia in Japanese black bears were much lower than those estimated for black bears in Alaska (32%) (Chomel et al., 1998) and Idaho (19%) (Binninger et al., 1980). It is not known whether the difference was due to the methods used in those studies.

There was a difference in the prevalence between male and female bears (Table 2). Although the number of samples is small, this finding may help understand the ecology of *F. tularensis* in Japanese black bears. It is known that male bears roam much broader area compared with females, suggesting that the difference in habitat or territory between genders (Tsubota et al., 1998) results in more frequent exposure of male bears to infectious agents including *F. tularensis*.

None of Japanese hares turned out be seropositive even those captured in the areas where human tularemia was reported. Although one sample appeared as positive in the screening assay (ELISA), subsequent confirmatory tests using WB and IFA indicated that the samples did not contain specific antibody to *F. tularensis*. Several bands observed when whole cell lysate was used as antigen in WB were probably due to non-specific reaction caused by unknown components in the sera or cross-reaction with the antibody to other bacteria. Because hares are highly susceptible to *F. tularensis* infection (Mörner and Addison, 2001), it is likely that a majority of infected hares die out before developing an immunological response to *F. tularensis* infection. Their short lifespan in natural environment or habitat may be another reason for the difficulty of finding seropositive hares. Similar findings have been reported on hares in Europe (Mörner et al., 1988; Frölich et al., 2003). Seropositive rabbits were not found in tularemia endemic area in North America either (Berrada et al., 2006). Thus, it is unlikely that hares and rabbits play any role as sentinels in conducting serosurveillance of tularemia; however, there would be an increased chance of isolation of *F. tularensis* if fresh carcasses of hares or rabbits were available (Park et al., 2009). When planning surveillance of tularemia in wild animals, the fact that various factors may affect the results should be borne in mind, in particular, their lifespan, susceptibility to the pathogen as well as assay methods.

It seems likely that Japanese black bears will serve as the sentinel to assess the possible risk of tularemia outbreaks. Because continuous sampling from an individual seems feasible, bears are more useful as the sentinel than other animals like raccoon dogs or skunks (Berrada et al.,

2006). Japanese black bear is a subspecies of Asiatic black bear, which widely distributed throughout southern Asia, northern China and far eastern Russia (IUCN Red List of Threatened Species, 2009. Version 2009.2; http://www.iucnredlist.org). Therefore, surveillance of other subspecies of bears will help understand the distribution of *F. tularensis* in these areas as well.

This study serologically showed that reservoir animals of *F. tularensis* are rare but definitely thrive in the north-eastern part of Japan, where four human cases of tularemia were reported in 2008 (Infectious Diseases Weekly Report Japan, 19 January 2009). Thus, hunters and veterinarians should be advised to take necessary precautions when treating wild animals because tularemia is maintained in mammalian reservoir animals and outbreaks in humans often parallel those in animal populations (Tärnvik et al., 1996). Further serosurveillance in wild animal species including bears is now in progress.

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References

Al Dahouk, S., K. Nockler, H. Tomaso, W. D. Splettstoesser, G. Jungersen, U. Riber, T. Petry, D. Hoffmann, H. C. Scholz, A. Hensel, and H. Neubauer, 2005: Seroprevalence of brucellosis, tularemia, and yersiniosis in wild boars (*Sus scrofa*) from north-eastern Germany. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 52, 444–455.

Berrada, Z. L., H. K. Goethert, and S. R. Telford 3rd, 2006: Raccoons and skunks as sentinels for enzootic tularemia. *Emerg. Infect. Dis.* 12, 1019–1021.

Binninger, C. E., J. J. Beecham, L. A. Thomas, and L. D. Winward, 1980: A serologic survey for selected infectious diseases of black bears in Idaho. *J. Wildl. Dis.* 16, 423–430.

CDC, 2008: Public health consequences of a false-positive laboratory test result for Brucella – Florida, Georgia, and Michigan, 2005. *MMWR Morb. Mortal. Wkly. Rep.* 57, 603–605.

Chomel, B. B., R. W. Kasten, G. Chappuis, M. Soulier, and Y. Kikuchi, 1998: Serological survey of selected canine viral pathogens and zoonoses in grizzly bears (Ursus arctos horribilis) and black bears (*Ursus americanus*) from Alaska. *Rev. Sci. Tech.* 17, 756–766.

De Swart, R. L., Y. Nur, A. Abdallah, H. Kruining, H. S. El Mubarak, S. A. Ibrahim, B. Van Den Hoogen, J. Groen, and A. D. Osterhaus, 2001: Combination of reverse transcriptase PCR analysis and immunoglobulin M detection on filter paper blood samples allows diagnostic and epidemiological studies of measles. *J. Clin. Microbiol.* 39, 270–273.

Ellis, J., P. C. Oyston, M. Green, and R. W. Titball, 2002: Tularemia. *Clin. Microbiol. Rev.* 15, 631–646.

Francis, E., and D. Moore, 1926: Identity of Ohara's disease and tularemia. *J. Am. Med. Assoc.* 86, 1329–1332.

Frölich, K., J. Wisser, H. Schmüser, U. Fehlberg, H. Neubauer, R. Grunow, K. Nikolaou, J. Priemer, S. Thiede, W. J. Streich, and S. Speck, 2003: Epizootiologic and ecologic investigations of European brown hares (*Lepus europaeus*) in selected populations from Schleswig-Holstein, Germany. *J. Wildl. Dis.* 39, 751–761.

Fujita, O., M. Tatsumi, K. Tanabayashi, and A. Yamada, 2006: Development of a real-time PCR assay for detection and quantification of *Francisella tularensis*. *Jpn. J. Infect. Dis.* 59, 46–51.

Fujita, O., A. Uda, A. Hotta, A. Okutani, S. Inoue, K. Tanabayashi, and A. Yamada, 2008: Genetic diversity of *Francisella tularensis* subspecies holarctica strains isolated in Japan. *Microbiol. Immunol.* 52, 270–276.

Grebenchtchikov, N., C. G. Sweep, A. Geurts-Moespot, A. Piffanelli, J. A. Foekens, and T. J. Benraad, 2002: An ELISA avoiding interference by heterophilic antibodies in the measurement of components of the plasminogen activation system in blood. *J. Immunol. Methods* 268, 219–231.

Hopla, C. E., 1974: The ecology of tularemia. *Adv. Vet. Sci. Comp. Med.* 18, 25–53.

Hotta, A., A. Uda, O. Fujita, K. Tanabayashi, and A. Yamada, 2007: Preparation of monoclonal antibodies for detection and identification of *Francisella tularensis*. *Clin. Vaccine Immunol.* 14, 81–84.

Magnarelli, L., S. Levy, and R. Koski, 2007: Detection of antibodies to *Francisella tularensis* in cats. *Res. Vet. Sci.* 82, 22–26.

Mörner, T., and E. Addison, 2001: Tularemia. Iowa State University Press, Iowa, USA.

Mörner, T., G. Sandström, R. Mattsson, and P. O. Nilsson, 1988: Infections with *Francisella tularensis* biovar palaearctica in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden. *J. Wildl. Dis.* 24, 422–433.

Ohara, Y., T. Sato, H. Fujita, T. Ueno, and M. Homma, 1991: Clinical manifestations of tularemia in Japan – analysis of 1,355 cases observed between 1924 and 1987. *Infection* 19, 14–17.

Ohara, Y., T. Sato, and M. Homma, 1996: Epidemiological analysis of tularemia in Japan (yato-byo). *FEMS Immunol. Med. Microbiol.* 13, 185–189.

Park, C. H., A. Nakanishi, H. Hatai, D. Kojima, T. Oyamada, H. Sato, N. Kudo, J. Shindo, O. Fujita, A. Hotta, S. Inoue, and K. Tanabayashi, 2009: Pathological and microbiological studies of Japanese Hare (*Lepus brachyurus* angustidens) naturally infected with *Francisella tularensis* subsp. holarctica. *J. Vet. Med. Sci.* 71, 1629–1635.

Porsch-Ozcurumez, M., N. Kischel, H. Priebe, W. Splettstosser, E. J. Finke, and R. Grunow, 2004: Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin. Diagn. Lab. Immunol.* 11, 1008–1015.

Sato, T., H. Fujita, Y. Ohara, and M. Homma, 1990: Microagglutination test for early and specific serodiagnosis of tularemia. *J. Clin. Microbiol.* 28, 2372–2374.

Shoemaker, D., A. Woolf, R. Kirkpatrick, and M. Cooper, 1997: Humoral immune response of cottontail rabbits naturally infected with Francisella tularensis in southern Illinois. *J. Wildl. Dis.* 33, 733–737.

Tärnvik, A., G. Sandström, and A. Sjöstedt, 1996: Epidemiological analysis of tularemia in Sweden 1931–1993. *FEMS Immunol. Med. Microbiol.* 13, 201–204.

Tsubota, T., N. Mizoguchi, and I. Kita, 1998: Ecological and physiological studies of the Japanese black bear, *Ursus thibetanus* japonicus. *Jpn. J. Zoo Wildl. Med.* 3, 17–24.

Uda, A., K. Tanabayashi, O. Fujita, A. Hotta, Y. Yamamoto, and A. Yamada, 2007: Comparison of whole genome amplification methods for detecting pathogenic bacterial genomic DNA using microarray. *Jpn. J. Infect. Dis.* 60, 355–361.

Yamazaki, K., and O. Inaba, 2009: Possible expansion of Japanese black bear distribution in the southern part of the Abukuma Mts. (Ibaraki, Fukushima and Tochigi Prefectures), central part of Japan. *Mammalian Science* 49, 257–261.

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Epidemiological Report

Molecular Epidemiological Study of *Bacillus anthracis* Isolated in Mongolia by Multiple-Locus Variable-Number Tandem-Repeat Analysis for 8 Loci (MLVA-8)

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SUMMARY: The incidence of anthrax, which is caused by *Bacillus anthracis*, in the human and animal population of Mongolia has increased recently, and control of this infection is a nationwide concern. In this study, 29 isolates obtained from animals and various regions in Mongolia from 2001 to 2007 were analyzed by performing multiple-locus variable-number tandem-repeat analysis for 8 loci (MLVA-8) to understand the genetic relationship between the Mongolian *B. anthracis* isolates. We found that all the Mongolian isolates can be classified into A3 cluster along with the Japanese and the Chinese *B. anthracis* isolates. Our data revealed that MLVA-8 is useful for studying the molecular epidemiology of the Mongolian *B. anthracis* isolates and would help characterize *B. anthracis* infections in Mongolia.

INTRODUCTION

In Mongolia, animal husbandry, involving seasonal migration of livestock for grazing, is one of the major economic activities; therefore, the control of anthrax is a nationwide concern with respect to both human and animal health. Mongolia is located in East and Central Asia and bordered by Russia to the north and China to the south, east, and west. Mongolia has a varied geography with regions such as the Gobi Desert in the south and the cold and mountainous regions in the north and west. As stated previously (1), the topography of a country influences the source of introduction and route of transmission of infectious diseases.

Fromg 1964 to 2008, 212 human anthrax cases were reported in 77 sums (administrative subdivisions in Mongolia), 17 provinces, and the capital city of Mongolia; the disease was fatal in 13 of these cases. Most of the patients had acquired the infection through contact with diseased livestock; however, in the recent years, transmission of the infection from soil and livestock barns has been increasing (Fig. 1) (2). From 1977 to 2008, 4,222 cases of anthrax in livestock were reported in Mongolia. The northern provinces, especially Khuvsgul and Zavkhan, are hyper-endemic (Fig. 1) (2).

Thus far, molecular characterization of Japanese (3), Chinese (4), and Korean (5) *Bacillus anthracis* isolates using multiple-locus variable-number tandem-repeat analysis for 8 loci (MLVA-8) has been reported (6). MLVA-8 has been widely applied for the genetic study of *B. anthracis* isolates in many countries. In this analysis, isolates are classified into cluster groups (A1, A2, A3, and B–E) as per the combination of repeat numbers. The strains from Asian countries have been found to mostly belong to the A3 cluster. However, molecular studies on Mongolian isolates have not yet been reported. Genetic analysis will not only help understand the epidemiologic links between outbreaks better but also facilitate the development of various measures to control anthrax in Mongolia. In this study, we found that all the 29 Mongolian *B. anthracis* isolates obtained from livestock and soil during the period of 2001–2007 belong to the A3 cluster.

MATERIALS AND METHODS

The 29 *B. anthracis* isolates were collected by and stored at the National Center for Infectious Diseases with Natural Foci (NCIDNF), Ulaanbaatar, Mongolia (Table 1). The isolates were collected from across the country so as to obtain isolates from diverse areas of Mongolia. In order to minimize the occurrence of spontaneous mutation, we selected relatively new isolates that were stored in stable preservation condition and had definite passage, with a history of less than 15 passages.

For DNA extraction, the *B. anthracis* isolates were inoculated into meat-peptone broth and incubated at 37°C overnight without shaking. Then, the isolates were grown on 0.5% sheep blood agar and incubated overnight at 37°C. Purified colonies from each plate were picked up with an inoculation loop and suspended in 1.5 ml microcentrifuge tubes containing 500 μl of sterile distilled water. The suspensions were then heated at 100°C

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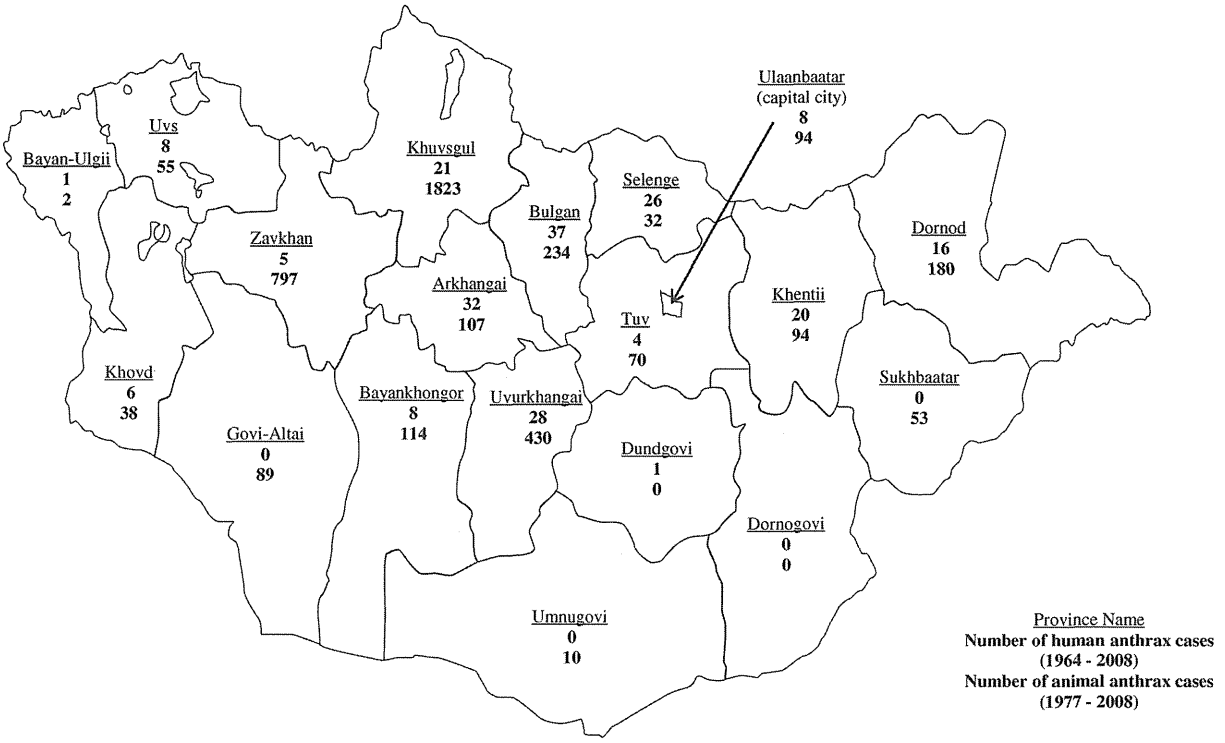

Fig. 1. Provinces of Mongolia with accumulative number of human anthrax cases of each province from 1964 to 2008 (upper column) and livestock from 1977 to 2008 (lower column) are shown.

Table 1. List of *Bacillus anthracis* strains of Mongolia with the number of tandem repeats by MLVA-8

Strain no.	Isolated province	Source	No. of tandem repeats by MLVA-8 determined in this study							
			vrrA	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	CG3	pXO1	pXO2
3	Khentii	soil	10	16	7	53	17	2	8	9
7	Uvurkhangai	soil	10	16	7	53	17	2	8	9
8	Uvurkhangai	soil	10	16	7	53	17	2	8	9
14	Uvurkhangai	sheep skin	10	16	7	53	17	2	8	9
16	Khentii	soil	10	16	7	53	17	2	8	9
17	Zavkhan	goat ear skin	10	16	7	53	17	2	8	9
24	Bulgan	cattle spleen	10	16	7	53	17	2	7	9
25	Dornod	soil	10	16	7	53	17	2	7	9
26	Khuvsgul	soil	10	16	7	53	21	2	8	9
27	Selenge	soil	10	16	7	53	17	2	8	9
4	Khentii	soil	10	16	7	53	17	2	8	10
6	Khentii	soil	10	16	7	53	17	2	8	10
21	Govi-Altai	soil	10	16	7	53	17	2	8	10
22	Khuvsgul	soil	10	16	7	53	17	2	8	10
23	Khuvsgul	sheep skin	10	16	7	53	17	2	8	10
1	Bayankhongor	goat brain	10	16	7	53	17	2	8	7
2	Ulaanbaatar	swab of meat counter	10	16	7	53	17	2	8	7
5	Khentii	soil	10	16	7	53	17	2	8	11
9	Bulgan	swab from patient cloth	10	16	7	53	17	2	8	11
10	Tuv	soil	10	16	7	53	17	2	8	11
11	Khentii	goat blood	10	16	7	53	17	2	8	11
12	Selenge	cattle skin	10	16	7	53	17	2	8	11
13	Uvurkhangai	soil	10	16	7	53	17	2	8	11
18	Govi-Altai	soil	10	16	7	53	17	2	8	7
19	Govi-Altai	soil	10	16	7	53	17	2	8	7
20	Govi-Altai	horse skin	10	16	7	53	17	2	8	7
28	Selenge	horse stomach contents	10	16	7	53	17	2	8	8
29	Bayankhongor	goat meat	10	16	7	53	17	2	8	8
30	Bayankhongor	cattle meat	10	16	7	53	17	2	8	11

for 15 min. The supernatant was used for DNA extraction using the Biospin Bacteria Genomic DNA Extraction kit (Bioer, Hangzhou, China); extraction was performed according to the manufacturer's instructions.

DNA fragments for 8 loci were obtained by performing PCR. DNA, 5 ng in a final volume of 50 μl with Platinum Taq DNA polymerase (Invitrogen Japan, Tokyo, Japan), and 0.1 μM of each primer was amplified using a Veriti Thermal Cycler (Life Technologies Japan, Tokyo, Japan). The reaction was carried out as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 68°C for 1 min. The reaction was terminated by a final extension at 68°C for 2 min. All loci were sequenced as described previously (3). Briefly, the PCR products were purified using the Wizard SV Gel and the Min-Elute purification kit (Qiagen Japan, Tokyo, Japan), and then direct sequencing was performed using a 3730xl DNA Analyzer (Life Technologies Japan), a Big Dye Terminator v3.11 Cycle Sequencing Kit (Life Technologies Japan), and appropriate primers. DNA sequences obtained from the multiple fragments were assembled and edited by GENETYX ver. 9 (GENETYX Corp., Tokyo, Japan).

The MLVA-8 results for 7 isolates from Japan (3), 14 from China, and 6 from Europe and Africa (4) were included in our data (see Fig. 2). The matrix distance was calculated and a dendrogram was drawn using unweighted pair-group method with arithmetic means (UPGMA) method using two programs, PHYLIP version 3.6 (http://evolution.genetics.washington.edu/phylip/phylipweb.html) and Tree View version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Nucleotide sequences of MLVA-8 of Mongolian *B. anthracis* isolates were registered with the DDBJ (accession no. AB597568–AB597581).

RESULTS AND DISCUSSION

Twenty-nine *B. anthracis* isolates with definite passage records were isolated grazing herbivores, soils, and the environments from across Mongolia and were analyzed by MLVA-8 (Table 1). Number of tandem repeats in the loci, *vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, and CG3 were identical among the tested Mongolian isolates. Only one isolate (No. 26) had 21 repeats in the *vrrC2* locus; 17 repeats were observed in case of the other isolates (Table 1). All the Mongolian isolates were classified into the A3 cluster along with the Japanese and Chinese isolates (Fig. 2). The cluster was divided into branches on the basis of the repeats of plasmids pXO1 and pXO2. The isolates obtained from hyper-endemic northern mountainous areas and provinces of Khuvsgul, Zavkhan, and Uvurkhangai, possessed 7 or 8 repeats of pXO1 and 9 repeats of pXO2. The isolates with 8 repeats of pXO1 and 10 repeats of pXO2 were from the northern mountains and the southern deserts. The isolates with 8 repeats of pXO1 and 7, 8, or 11 repeats of pXO2 were collected from the desert areas, Govi-Altai, Bayankhongor, and Ulaanbaatar (Table 1). These results suggest that the genetic sequences of the *B. anthracis* strains were conserved; however, genetic differences associated with the topographical distribution of the strains exist. In Mongolia, the northern mountainous areas, such as

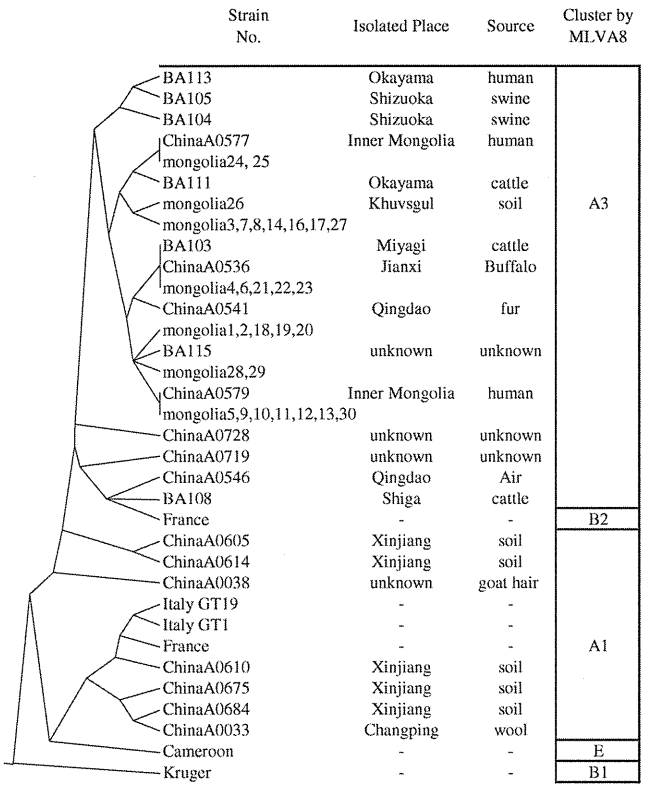

Fig. 2. Dendrogram constructed according to the MLVA-8 of *B. anthracis* strains including Mongolian isolates. The tree was drawn using UPGMA method as previously reported (3).

Table 2. Reported anthrax cases of livestock animals in mountainous (Khuvsgul and Zavkhan), desert (Govi-Altai), and city (Bulgan, Selenge, and the capital city Ulaanbaatar) areas

Province	1970s	1980s	1990s	2000–2008
Khuvsgul	145	573	785	320
Zavkhan	97	344	254	102
Govi-Altai	5	45	0	39
Bulgan	61	98	7	59
Selenge	0	1	10	21
Ulaanbaatar	0	2	52	40

Khuvsgul and Zavkhan have been highly endemic for anthrax in humans and animals (Fig. 1 and Table 2). However, since the 1990s, the number of livestock and population has increased in the urban areas and big cities in Selenge and Bulgan provinces and Ulaanbaatar, because of which anthrax outbreaks have been more frequently reported in these areas (Table 2). This finding indicates that the recent changes in the economic and social behavior of the population should be considered in the epidemiology of anthrax in Mongolia.

ChinaA0577 and ChinaA0579 (4), which were isolated from Inner Mongolia, were grouped into the same branches as some Mongolian isolates (Fig. 2). As per our study, genetically similar bacterial strains appear to circulate in topologically related areas. However, the BA103 (3) strain isolated in Japan and the ChinaA0536 (4) strain isolated from Jianxi, a central area in China, were also clustered into one branch together with some Mongolian isolates. Furthermore, soil seems to be an

important source and route of anthrax infection in Mongolia because 55% of the *B. anthracis* isolates analyzed in our study were isolated from soils from different areas. Since the data presented here are limited, it is necessary to continue epidemiological monitoring and surveillance of human and animal clinical isolates and their environment (including soil). The strain-level distinction of human and animal clinical isolates as well as environmental isolates can be achieved by single nucleotide polymorphism (SNP) analyses such as the 80-tag SNP typing because these techniques afford high-resolution genotyping power (7).

Further molecular epidemiological studies with adequate monitoring and surveillance would help establish the etiology of *B. anthracis* in Mongolia, and thereby, help initiate strategies to implement anthrax control measures.

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Conflict of interest None to declare.

REFERENCES

1. Boldbaatar, B., Inoue, S., Tuya, N., et al. (2010): Molecular epidemiology of rabies virus in Mongolia, 2005–2008. Jpn. J. Infect. Dis., 63, 358–363.
2. Burmaa Kh, U.B., Batbaatar, E., Uranshagai, N., et al. (2009): Geographical Distribution of Anthrax in Mongolia. Proceedings of Zoonotic Infectious Diseases and Tourism.
3. Okutani, A., Sekizuka, T., Boldbaatar, B., et al. (2010): Phylogenetic typing of *Bacillus anthracis* isolated in Japan by multiple locus variable-number tandem repeats and the comprehensive single nucleotide polymorphism. J. Vet. Med. Sci., 72, 93–97.
4. Van Ert, M.N., Easterday, W.R., Huynh, L.Y., et al. (2007): Global genetic population structure of *Bacillus anthracis*. PLoS ONE, 2(5), e461.
5. Ryu, C., Lee, K., Hawng, H.J., et al. (2005): Molecular characterization of Korean *Bacillus anthracis* isolates by amplified fragment length polymorphism analysis and multilocus variable-number tandem repeat analysis. Appl. Environ. Microbiol., 71, 4664–4671.
6. Keim, P., Price, L.B., Klevytska, A.M., et al. (2000): Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. J. Bacteriol., 182, 2928–2936.
7. Kuroda, M., Serizawa, M., Okutani, A., et al. (2010): Genome-wide single nucleotide polymorphism typing method for identification of *Bacillus anthracis* species and strains among *B. cereus* group species. J. Clin. Microbiol., 48, 2821–2829.

Ⅳ. 平成24年度総括・分担研究報告書

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）
総括研究報告書

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

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研究要旨　本研究では「One Health」理念を念頭に置きつつ、分野横断的なアプローチにより、動物由来感染症の制御に深く関連する、診断、予防、治療について研究を深めること並びにこれらの病原体の病原性発揮の機構を知ることを目的とし、以下の成績を得た。

① 本年度は、昨年に引き続き岡山県 T と畜場へ 2011 年から 2012 年に全国から搬入された食用ウシ 299 頭を対象に、*C. burnetii* に対する抗体を測定したところ、21 頭が陽性を示した。また、遺伝子疫学調査として、T と畜場へ 2012 年に搬入された食用ウシ 150 頭、北海道の牧場ウシ 205 頭、北海道の牧場ウマ 87 頭、岡山県のヌートリア 148 頭、岡山県の野ネズミ 133 頭、岡山県のマダニ 180 匹及び北海道のマダニ 563 匹を対象として *C. burnetii* の遺伝子検索を実施したが、結果は全て陰性であった。

② 国内でのライム病ボレリアの高感度 DNA 型別解析法による遺伝子データベース構築作業を継続し、その解析結果から国内患者由来株の大多数を占める *Borrelia garinii* では, ST128, ST131, ST362 の 3 つの ST 型 43%を占有すること、またこれら ST は自然界では *Myodes rufocanus bedfordiae, Apodemus speciosus, A. argenteus* から見出されることが明らかとなった。また、猟犬を歩哨動物としたライム病ボレリアの血清疫学調査を行い、シュルツェマダニが生息する東北地方ではウエスタンブロット法により 48.8%の猟犬がライム病抗体陽性と判定された。北信越地方以南の抗体陽性率は 23.5%以下であることから、猟犬を歩哨動物とする血清疫学調査は、病原体浸潤地域を調べる上で有用であると考えられる。

③ 昨年度までに野生イノシシにブルセラ菌に対する凝集抗体の存在が認められたが、他の菌との交差反応である可能性も否定できないため、より特異的な抗体検出法の開発を試みた。ブルセラの n-lauroylsarcosine 抽出（SE）抗原を用いた WB 法でブルセラ免疫ウサギ血清と特異的に反応する 11kDa 付近に見られるバンドを抽出し、そのアミノ酸ならびに遺伝子配列を決定したところ、hypothetical protein BMEI0805 であることが明らかとなった。pCold TF ベクターを用いて組換えタンパクとして発現させ、その特異性を検討したところ、感染イヌ血清については十分な本能性を示した。しかしブルセラ属菌以外のグラム陰性菌で免疫したウサギ血清と大腸菌由来のタンパクによるものと考えられる非特異的反応も見られた。また、TF（Trigger factor）を除去したベクターも作成し検討を加えたが、免疫ウサギ血清の非特異的反応の除去には至らなかった。そこで、あらためて SE 抗原を二次元電気泳動により展開したところ、サイズが同一で PI 値の異なる（PI 値=8）タンパクの存在が明らかになった。しかし PI 値=6 付近にブルセラ感染イヌ血清と特異的に反応するタンパクが認められたことから、本タンパクに焦点を絞り検討を継続している。

④ 新たにジフテリア様症状を呈する 2 名の患者からジフテリア毒素産生性 *Corynebacterium ulcerans* (*C.ulcerans* $^{Tox+}$)を分離した。患者の環境調査において飼いネコ及びイヌから *Corynebacterium* 属菌が分離された。また、患者家族のジフテリア抗毒素抗体価が高かった。これらの症例は、今まで報告されたような呼吸器症状ではなくリンパ節膿瘍や皮下膿瘍の症例であった。動物愛護センターや動物病院に搬入されたイヌまたはネコの咽頭スワブ等について菌分離調査及び血清ジフテリア抗毒素抗体価測定を実施した結果、愛護センターのネコとイヌより *C.ulcerans* $^{Tox+}$8株が分離され、28 検体が抗毒素陽性を示した。

⑤ 東北地方（福島県、宮城県、岩手県、青森県、秋田県および山形県）の猟犬計 123 頭について、ジフテリア症、破傷風、ブルセラ症、トキソプラズマ症、レプトスピラ症およびボレリア症に関する調査を実施した。ブルセラ症は福島で 1 例、山形県で 3 例の計 4 例の陽性例が認められた。トキソプラ

ズマでは、これまでと同様に各県とも高い陽性率で 18%が陽性を示した。ジフテリア毒素では、青森で 1 例の陽性例が認められた。東北地方で問題となる野兎病は、今回検出されなかった。犬由来の感染で、宮城県で *Babesia (B) gibsoni* が 3 例にのみ検出された。また、ヘパトゾーン(*Hepatozoon canis*)について、全例とも陰性であった。犬フィラリア症では、検索した猟犬全体の約30%で抗原が検出され、比較的高い陽性率であった。

⑥ これまで野兎病菌 LPS および抗 LPS モノクローナル抗体を用いた競合 ELISA (cELISA)の開発を試みてきたが、今年度は、野兎病患者血清について本 cELISA と間接 ELISA (iELISA)との相関性を検証するとともに、国内野生動物の血液 632 検体について cELISA および微量凝集反応(MA) 法による野兎病菌抗体の検出を試みた。ヒト血清を cELISA と iELISA で調べた結果、両者は高い相関を示し、ヒト血清抗体の測定に応用可能なことが明らかとなった。また、野生動物血液検体を cELISA で調べた結果、ツキノワグマ(23/150)、ホンドタヌキ(3/21)で陽性個体が認められた。MA 法で検出されなかった検体にも cELISA 陽性検体が見いだされることから、cELISA は MA 法より高感度であることが確認された。動物種特異的標識二次抗体等を用いる必要のない本法は、野生動物における野兎病の血清疫学調査のための手法として有用と考えられた。

⑦ 野兎病菌の病原性の分子的基盤を解析するために弱毒株および強毒株の全ゲノムを比較した結果、野兎病菌ゲノム 190 万塩基の中で 1 塩基のみが異なることが明らかになった。この 1 塩基の違いにより強毒性株では正常 PdpC タンパク質を発現できるのに対し、弱毒性株では約半分の大きさの異常型 PdpC タンパク質しか発現できない事が明らかとなり、PdpC タンパク質が病原性に関与している可能性が示唆された。そこで、強毒性株の *pdpC* 遺伝子破壊株およびその破壊株にプラスミドで *pdpC* 遺伝子を相補した株（相補株）を新たに作出し、マウスを用いて病原性を評価した結果、*pdpC* 遺伝子破壊株は病原性を消失したが、その相補株は病原性が復帰していた。以上の結果より、野兎病菌 *pdpC* 遺伝子は極めて重要な病原性を規定する遺伝子であることが明らかとなった。

⑧ 昨年度開発したマイクロアレイでの解析に至適化した *in vitro* の人工培養系を用い、エキノコックス症の感染源となるステージである成虫型へ発育分化を誘導した材料を用意した。これをマイクロアレイによるトランスクリプトーム解析に供することで、処理にともなって特徴的に発現する遺伝子群を同定した。また、遺伝子発現量の変化を測定することで、分化の決定がなされる時期を明らかにした。

⑨ 狂犬病ウイルス（RABV）抗原を特異的に認識する single chain variable fragment (scFv)を使用した Direct Rapid Immunohistochemical Test (DRIT)法の確立を目的とし、RABV 蛋白質を特異的に認識するビオチン融合タンパク化した single chain variable fragment (scFv)によるより簡易な DRIT の開発を検討した。これまでに、ビオチンを融合した抗 RABV-P タンパク scFv を発現できるクローン（scFv-P19-biotin）の作出に成功した。現在、大腸菌で発現させた scFv-P19-biotin タンパクを利用した DRIT 法の確立を進めている。

⑩ 海外の狂犬病常在地にて動物咬傷を受傷し、狂犬病ワクチン接種歴がない 18 例を対象とし、外国産（9 例）及び国内産（9 例）ワクチンを用いた 4 回接種による暴露後免疫の効果を検討した。狂犬病抗体幾何平均は 1.36EU/mL、抗体陽転率（有意抗体価 0.5EU/mL 以上）は 89%であった。狂犬病は発症すると致死的であり、暴露後免疫により 100%に限りなく近い抗体陽転率が求められることから、現時点において 4 回接種法は容認しがたいと思われた。

⑪ 日本国内には、狂犬病の治療、院内感染対策に関する資料が非常に乏しいことが明らかとなったため、今年度は狂犬病治療を考える基礎資料として海外から報告された文献に基づき、狂犬病救命例、治療法、院内感染対策についてまとめた。

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A.　研究目的

動物由来感染症は世界に 200 以上存在し、その病原体は 850 種を超える。新興感染症の殆どは動物由来感染症であるのみならず、現時点でヒトに特化した感染症もすべてが動物に由来すると言っても過言ではない。動物由来感染症は自然生態系との関与が大きく、その制御にはヒトへの視点からのみでなく、家畜、野生動物さらにはそれらを取り巻く環境への視点が欠かせない。近年提唱されている「One Health」の基本的考え方である。本研究では「One Health」理念を念頭に置きつつ、分野横断的なアプローチにより、動物由来感染症の制御に深く関連する、診断、予防、治療について、これまで対象としてきた感染症を中心に研究を深める。一方で国内では稀となったり、その存在がはっきりと確認されてなかったような動物由来感染症について、その実態を明らかにすべく、モニタリングあるいはサーベイランスを実施する。具体的にはライム病、ブルセラ症、野兎病、Q 熱、コリネバクテリウムウルセランス感染症、エキノコックス症、狂犬病について、これらの研究を実施する。また、野生動物に接触する機会の多い職業に従事する者の血清疫学調査を行い、リスクを評価する。これらの研究によって国内に存在する動物由来感染症に関する情報の整備が可能となり、これにより新興感染症の発生があった場合にいち早く検出することが可能になると考えられる。

B.　研究方法

病原体あるいは抗体の検出は個々の報告書に記載した方法による。

C.　研究結果

（1）Q 熱に関する研究

Q 熱は、感染症法では 4 類感染症に分類される人獣共通感染症であるが、ヒトでは特異的な症状が認められず、インフルエンザ様疾患、肺炎、肝炎等、多彩な病状を示す一方、動物では一般に無症状とされる。感染症発生動向調査事業における患者数は 2002 年から 2003 年がピークで、それぞれ 40 人を超えていた。しかし、近年は発生が極めて稀で、起因菌である *Coxiella burnetii* の国内における実態は未だ不明な点が多い。そこで、本病原体の侵淫状況を明らかにし、ヒトへの感染リスクを評価するため、これまでにヒト、伴侶動物であるイヌ、ネコ、家畜であるウシ、さらに宿主と目されるマダニを対象に疫学調査を実施してきた。その結果、これらの抗体保有率及び *C. burnetii* 遺伝子の検出率は、1990 年代の報告と比較して全て低値を示した。

今回、血清疫学調査として、昨年に引き続き岡山県 T と畜場へ 2011 年から 2012 年に全国から搬入された食用ウシ 299 頭を対象に、*C. burnetii* に対する抗体を測定したところ、21 頭が陽性を示した。また、遺伝子疫学調査として、T と畜場へ 2012 年に搬入された食用ウシ 150 頭、北海道の牧場ウシ 205 頭、北海道の牧場ウマ 87 頭、岡山県のヌートリア 148 頭、岡山県の野ネズミ 133 頭、岡山県のマダニ 180 匹及び北海道のマダニ 563 匹を対象として *C. burnetii* の遺伝子検索を実施したが、結果は全て陰性であった。

2009 年から 2012 年まで、全国の食用ウシ 565 頭を対象として血清疫学調査及び遺伝子疫学調査を実施してきたが、*C. burnetii* に対する抗体保有率は 3.7%であり、遺伝子は検出されなかった。これらの結果から、食用ウシにおいては、過去の感染が疑われる個体が存在するものの、調査時点では感

染個体はいなかったと考えられた。これまでに、遺伝子疫学調査として家畜 1313 頭、ペット 2748 頭、野生動物 281 頭及びマダニ 1365 匹の合計 5,707 検体について *C. burnetii* の遺伝子検索を実施してきたが、感染個体は確認できなかった。これらのことを勘案すると、現時点において、国内の *C. burnetii* の侵淫度は低く、ヒトへの感染リスクは非常に低いものと考えられた。しかしながら、現在も毎年数名の患者が報告されており、海外の流行地域からの輸入感染も懸念されることから、今後も検査体制やサーベイランス体制を維持していくことが必要であると考える。

（2）ボレリアに関する研究

1) 患者由来株(33 株)で優先する ST は ST131(22%)、ST128（12%)、ST362(9%)であり、これら主要 3ST で患者分離株全体の 43%を占有することが明らかとなった。

2) マダニ由来株(30 株)では、患者由来株と同一の ST(ST127、 ST371、ST375、ST385)をしめす 6 株が *I. persulcatus* より分離された一方で、残り 24 株は患者由来株とは一致しない ST であった。特に *I. pavlovski* より分離された 10 株はすべて患者由来株とは異なる ST であった。

3) 野鼠由来株(25 株)の内、18 株(72%)は患者由来株と同一 ST であった。特に患者由来株の主要 3 ST である ST131、ST128、ST362 の野鼠分離株での占有率は、*Myodes rufocanus bedfordiae* で 56%、*Apodemus speciosus* で 33%、*A. argenteus* で 25%であった。

4) 猟犬血清 121 検体のうち、ELISA 法にて 106 検体(87.6%)が抗ボレリア IgG 抗体陽性もしくはボーダーラインと判定され、うち 59 検体(48.8%)が WB 法により陽性であることが確認された。昨年度までに抗ボレリア抗体陽性と判定された検体は東北以外の地域では 16.4%であり（H23 年度分担報告書参照）東北地方では猟犬の抗ボレリア IgG 抗体陽性率は有意に高いことが示された。培養によるイヌ全血からのボレリア検出は陰性だった。

（3）コリネバクテリウムに関する研究

新たにジフテリア様症状を呈する 2 名の患者からジフテリア毒素産生性 *Corynebacterium ulcerans*（*C.ulcerans* Tox+）が分離された。患者の環境調査において飼いネコ及びイヌから *Corynebacterium* 属菌が分離された。また、患者家族のジフテリア抗毒素抗体価が高かった。これらの症例は、今まで報告されたような呼吸器症状ではなくリンパ節膿瘍や皮下膿瘍の症例であった。

12 カ所の地方自治体の動物愛護センターや動物病院に搬入されたイヌまたはネコの咽頭スワブ等について菌分離調査及び血清ジフテリア抗毒素抗体価測定を実施した結果、4 カ所の愛護センターのネコとイヌより *C.ulcerans* Tox+ 8 株が分離され、また抗毒素は 5 箇所 28 検体が陽性を示した。

今年度までの調査結果では、野外活動時間の多いイヌやネコは本菌を保菌または本菌に感染している可能性が懸念される。しかも外見上健康な動物が保有していた。感染した動物からは動物への菌の伝播がおこり感染が成立する。感染動物では排菌量が多いために、人はイヌ、ネコからの感染リスクが高いことが考えられる。

（4）ヒトの動物由来感染症への曝露の指標としての猟犬の応用に関する研究