皮質活動の欠如（脳波）	14-24	経頭蓋ドップラー超音波 緊急 CT±CT 血管造影
上大静脈血栓	22	ヘパリン 弾性ストッキング

表 3　MRP Version3.1 の要点

＜支持療法＞

・カテコラミン性神経伝達物質の生成に必要な tetrahydrobiopterin（BH4）の補充は、全例には行わず、低血圧、脳血管痙縮などが認められる際に使用される。

・全身管理における麻酔深度の調整

・脳死の診断基準を当てはめることはできず、鑑別に脳生検、脳血流測定を要する

・脳血管痙縮予防のため、カルシウム拮抗薬（Nimodipine 国内未承認薬）を使用する。

＜神経保護＞

・神経保護は、主にベンゾジアゼピン、ケタミン、アマンタジンにより行われる

・フェノバルビタールは、神経障害の惹起、免疫応答を抑制する懸念があり、全例には投与せず、必要時のみに使用する。

＜抗ウイルス療法＞

・狂犬病ウイルスのクリアランスは生体の免疫反応により行われる

・発症後の抗狂犬病免疫グロブリン、狂犬病ワクチンの投与は、免疫を修飾し病勢を悪化させる可能性があり禁忌とする

・リバビリンの効果は不明であり、溶血性貧血などの副作用を有することや、免疫応答を抑制し臨床症状を悪化させる可能から、禁忌とされた。

表 4　狂犬病治療における院内感染対策　32）33）

・狂犬病患者の診療・看護に直接関わる場合には、ガウン、ゴーグル、マスク、グローブ（Personal protective equipment PPE）の着用を要する

・感染リスクのある医療スタッフに対して、曝露前免疫の実施を考慮

・剖検、生検、局所解剖を実施する際は、適切な PPE の着用を要する

・汚染された器具は、高圧滅菌消毒または煮沸消毒を要する

・遺体は防腐処置を行わず、早期に火葬または埋葬する

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻　号	ページ	出版年
Takano A, Nakao M, Masuzawa T, Takada N, Yano Y, Ishiguro F, Fujita H, Ito T, Ma X, Oikawa Y, Kawamori F, Kumagai K, Mikami T, Hanaoka N, Ando S, Honda N, Taylor K, Tsubota T, Konnai S, Watanabe H, Ohnishi M, Kawabata H.	Multilocus sequence typing implicates rodents as the main reservoir host of human pathogenic *Borrelia garinii* in Japan.	Journal of Clinical Microbiology	49	2035-2039	2011
Katsukawa C, Komiya T, Yamagishi H, Ishii A, Nishino S, Nagahama S, Iwaki M, Yamamoto A, Takahashi M.	Prevalence of *Corynebacterium ulcerans* in dogs in Osaka, Japan.	J Med Microbiol.	61	266-273	2012
Hotta A., Tanabayashi K., Yamamoto Y, Fujita O, Uda A, Mizoguchi T and Yamada A.	Seroprevalence of tularemia in wild bears and hares in Japan.	Zoonoses and Public Health	59	89-95	2012
Okutani A, Tungalag H, Boldbaatar B, Yamada A, Tserennorov D, Otgonchimeg I, Erdenebat A, Otgonbaatar D, and Inoue S.	Molecular Epidemiological Study of *Bacillus anthracis* Isolated in Mongolia by Multiple-Locus Variable-Number Tandem-Repeat Analysis for 8 Loci (MLVA-8)	Japanese Jouranl of Infectious Diseases	64	345-348	2011

Ⅳ. 研究成果の刊行物・別刷

JOURNAL OF CLINICAL MICROBIOLOGY, May 2011, p. 2035–2039 Vol. 49, No. 5
0095-1137/11/$12.00 doi:10.1128/JCM.02544-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Multilocus Sequence Typing Implicates Rodents as the Main Reservoir Host of Human-Pathogenic *Borrelia garinii* in Japan▽†

Ai Takano,[1] Minoru Nakao,[2] Toshiyuki Masuzawa,[3] Nobuhiro Takada,[4] Yasuhiro Yano,[4] Fubito Ishiguro,[5] Hiromi Fujita,[6] Takuya Ito,[7] Xiaohang Ma,[8] Yozaburo Oikawa,[9] Fumihiko Kawamori,[10] Kunihiko Kumagai,[11] Toshiyuki Mikami,[11] Nozomu Hanaoka,[12,13] Shuji Ando,[13] Naoko Honda,[1,14] Kyle Taylor,[15] Toshio Tsubota,[15] Satoru Konnai,[15] Haruo Watanabe,[1] Makoto Ohnishi,[1] and Hiroki Kawabata[1]*

Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[1]; Department of Parasitology, Asahikawa Medical College, Asahikawa 078-8510, Hokkaido, Japan[2]; Faculty of Pharmacy, Chiba Institute of Science, Chiba 288-0025, Japan[3]; Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan[4]; Fukui Prefectural Institute of Public Health and Environmental Science, Fukui 910-8551, Japan[5]; Ohara Research Laboratory, Ohara General Hospital, Fukushima 960-0195, Japan[6]; Department of Biological Science, Hokkaido Institute of Public Health, Hokkaido 060-0819, Japan[7]; Department of Microbiology, College of Life Science, Zhejiang University, Hangzhou 310058, People's Republic of China[8]; Department of Medical Zoology, Kanazawa Medical University, Ishikawa 920-0293, Japan[9]; Department of Microbiology, Shizuoka Institute of Environment and Hygiene, Shizuoka 420-8637, Japan[10]; Department of Microbiology, Aomori Prefectural Institute of Public Health and Environment, Aomori 030-8566, Japan[11]; Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[12]; Department of Virology I, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[13]; Division of Radiological Protection and Biology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan[14]; and Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan[15]

Received 15 December 2010/Returned for modification 16 January 2011/Accepted 3 March 2011

Multilocus sequence typing of *Borrelia garinii* isolates from humans and comparison with rodent and tick isolates were performed. Fifty-nine isolates were divided into two phylogenetic groups, and an association was detected between clinical and rodent isolates, suggesting that, in Japan, human-pathogenic *B. garinii* comes from rodents via ticks.

Lyme disease is a multisystemic disorder caused by infection with the tick-borne spirochetes *Borrelia burgdorferi* sensu lato (s.l.). *B. burgdorferi* sensu stricto (s.s.), *Borrelia garinii*, and *Borrelia afzelii*, which are the known pathogenic borreliae of humans. *B. burgdorferi* s.s. is geographically distributed throughout North America and Europe, whereas *B. garinii* and *B. afzelii* are distributed throughout Europe and Asia. These *Borrelia* species are transmitted by *Ixodes ricinus* in Europe and *Ixodes persulcatus* in Asia and Russia. (2, 16, 24). In Europe, serological characterization has revealed that *B. garinii* is composed of several OspA serotypes (27). At present, it is understood that one *B. garinii* serotype (*B. garinii* OspA serotype 4) is maintained by rodents (7), although other serotypes of *B. garinii* are maintained by birds (4, 5, 26). Strains classified into *B. garinii* OspA serotype 4 were found to be distinguishable from other *B. garinii* strains by multilocus sequence typing (MLST), which was recently established for Lyme disease borreliae (14, 15). In Japan, *B. garinii* is known to be the main pathogenic borrelia, and it is transmitted by *I. persulcatus* (29). However, the natural reservoir host of human pathogenic *B. garinii* remains unclear since *I. persulcatus* infests both rodents and birds in Japan (28). To resolve this question, MLST analysis was performed on clinical, tick, and rodent isolates, and phylogenetic relationships among these strains were investigated.

Nineteen *B. garinii* strains were obtained for MLST analysis from Lyme disease patients with erythema migrans in Japan. Human isolates were cultured from erythema migrans lesions as previously described (22). As for tick and rodent isolates, 40 strains were examined. The sources of these strains are listed in Table 1. Eighteen strains were isolated from *I. persulcatus* ticks, which were collected from Japan (15 strains) and Russia (3 strains). Twenty-two strains were isolated from rodents. Of these, 10 were from *Myodes rufocanus* subsp. *bedfordiae* and 8 from *Apodemus speciosus* (both sets collected in Japan), and 4 were from *A. uralensis* (collected in China) (Table 1). The cultivation of borreliae was carried out at 34°C in modified Barbour-Stoenner-Kelly (BSK) medium (using minimal essential medium alpha [BioWest, Germany] as a substitute for CMRL-1066) (1). These strains were stored at −80°C until use. Cultivated bacterial cells (late-log phase) were used in DNA preparation. The genomic DNA of isolated strains was prepared by using a DNA extraction kit (DNeasy blood and tissue kit; Qiagen, Germany) according to the manufacturer's instructions. The PCR assay was performed according to Margos et al. (14, 15). After DNA amplification of eight loci (*clpA*, ATP-dependent Clp protease subunit A gene; *clpX*, ATP-dependent Clp protease subunit X gene; *nifS*, aminotransferase gene; *pepX*, dipeptidyl aminopeptidase gene; *pyrG*, CTP synthase; *recG*, DNA recombinase gene; *rplB*, 50S ribosomal protein L2 gene; and *uvrA*, excinuclease ABC subunit A gene),

* Corresponding author. Mailing address: Department of Bacteriology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1163. E-mail: address: kbata@nih.go.jp.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 16 March 2011.

TABLE 1. *Borrelia garinii* strains used in this study

B. garinii strain (no. of isolates)	Isolation source	Location	Reference
Tick isolates (18)			
HP1, HP3, HT18, HT59, N346, HkIP1, HkIP2	*Ixodes persulcatus*	Hokkaido, Japan	3, 10, this study
NP4, NP8, NP76, NP81, NT24, NT25, NT31	*I. persulcatus*	Nagano, Japan	10, 17
FujiP2	*I. persulcatus*	Shizuoka, Japan	11
Ip90	*I. persulcatus*	Khabarovsk, Russia	23
Mp7, Np189	*I. persulcatus*	Moscow, Russia	18
Rodent isolates (22)			
Ear isolates (17)			
HkCR1, HkCR3, HkCR4, HkCR5, HkCR6, HkCR7, HkCR9, HkCR11, HkCR12,	*Myodes rufocanus* subsp. *bedfordiae*	Hokkaido, Japan	9
FsAE1, FsAE2	*Apodemus speciosus*	Fukushima, Japan	8
FiEE11	*A. speciosus*	Fukui, Japan	8
sai8E	*A. speciosus*	Aomori, Japan	This study
ChYAE2	*A. uralensis*	Yakeshi, China	13
CTA1b, CTA4a, CTA5b	*A. uralensis*	Urumqi, China	25
Spleen isolate (1)			
ASF	*A. speciosus*	Hokkaido, Japan	19
Bladder isolates (4)			
HokkaidoCRB35B	*M. rufocanus* subsp. *bedfordiae*	Hokkaido, Japan	This study
HokkaidoAS7B	*A. speciosus*	Hokkaido, Japan	This study
sai6B, sai7B	*A. speciosus*	Aomori, Japan	This study
Human skin isolates (19)			
Hiratsuka	Erythema migrans	Niigata, Japan	This study
J-14, J-15, J-16, J-17, J-18, J-20T, J-21, J-32, J-33, J-34, J-35, J-37, J-38, J-39, J-40, J-41, J-42	Erythema migrans	Hokkaido, Japan	Miyamoto et al., unpublished data
HH1	Erythema migrans	Hokkaido, Japan	Sato et al., unpublished data

PCR products were purified by using ExoSAP-IT (GE Healthcare UK, Ltd., United Kingdom) and were directly sequenced (ABI Prism 3130xl Genetic Analyzer; Life Technologies Corporation). All sequences were deposited in GenBank (see the table in the supplemental material). In addition, reference sequences of each sequence type (ST) were downloaded from the MLST website (www.mlst.net). After concatenation of the sequences, Bayesian phylogenetic inference was performed (15). The phylogenetic tree was created according to Margos et al. (15), using TreeView software (ver. 1.6.6).

Isolated *B. garinii* strains were classified into two phylogenetic groups (preliminarily designated *B. garinii* ST group A and ST group B) by analysis of the concatenated DNA sequences of 8 loci (Fig. 1 and Table 2). The results indicated that *B. garinii* ST group B contained most of the Japanese clinical isolates (16/19 [84.2%]), all of the rodent isolates from Japan (18/18 [100%]) and China (4/4 [100%]), and 5 isolates from *I. persulcatus* collected in Japan (4/15 [26.7%]) and Russia (1/3 [33.3%]). Chi-square analysis indicated a confidence level of over 99% that *B. garinii* ST group B is predominant among Japanese clinical isolates and rodent isolates.

In this study, all *B. garinii* isolates from rodents were included in ST group B. In Europe, a recent report designated STs 84 and 85 as "*Candidatus* Borrelia bavariensis," and the reservoir host was thought to be rodents (14). Since *B. garinii* STs 84 and 85 clustered with *B. garinii* ST group B, we hypothesized that rodents are the main reservoir host of this phylogenetic group. In this study, the STs of 9 clinical isolates (2 of ST128, 4 of ST131, and strains J-14, J-18, and J-41) were found among rodent isolates. Thus, it can be inferred that human-pathogenic *B. garinii* is maintained by rodents in Japan. *B. garinii* ST group B was also found among rodent isolates from China. In addition, STs 128 and 131 of *B. garinii*, which were originally recorded in the MLST database as Chinese isolates, are pathogenic to humans in Japan. These suggest that *B. garinii* ST group B may also represent a health threat of Lyme

FIG. 1. Bayesian phylogenetic inference of concatenated housekeeping gene sequences of *B. garinii*. The phylogenetic tree was constructed based on Bayesian phlylogenetic inference. The posterior probability values of the clades are provided. Bars labeled 0.05 depict 5% divergence. *B. garinii* human isolates are indicated in red, rodent isolates in green, and isolates from *I. persulcatus* in blue. The relapsing fever *Borrelia* spp. (*B. duttonii* Ly [NC_011229], *B. hermsii* DAH [NC_010673], *B. recurrentis* A1 [NC_011244], and *B. turicatae* 91E135 [NC_008710]) were used as outgroups (data not indicated). The accession numbers of alleles from *Borrelia bissettii* DN127, *Borrelia andersonii* 21123, *Borrelia sinica* CMN3, and *Borrelia spielmanii* PsigII 27/6 are listed in the table in the supplemental material.

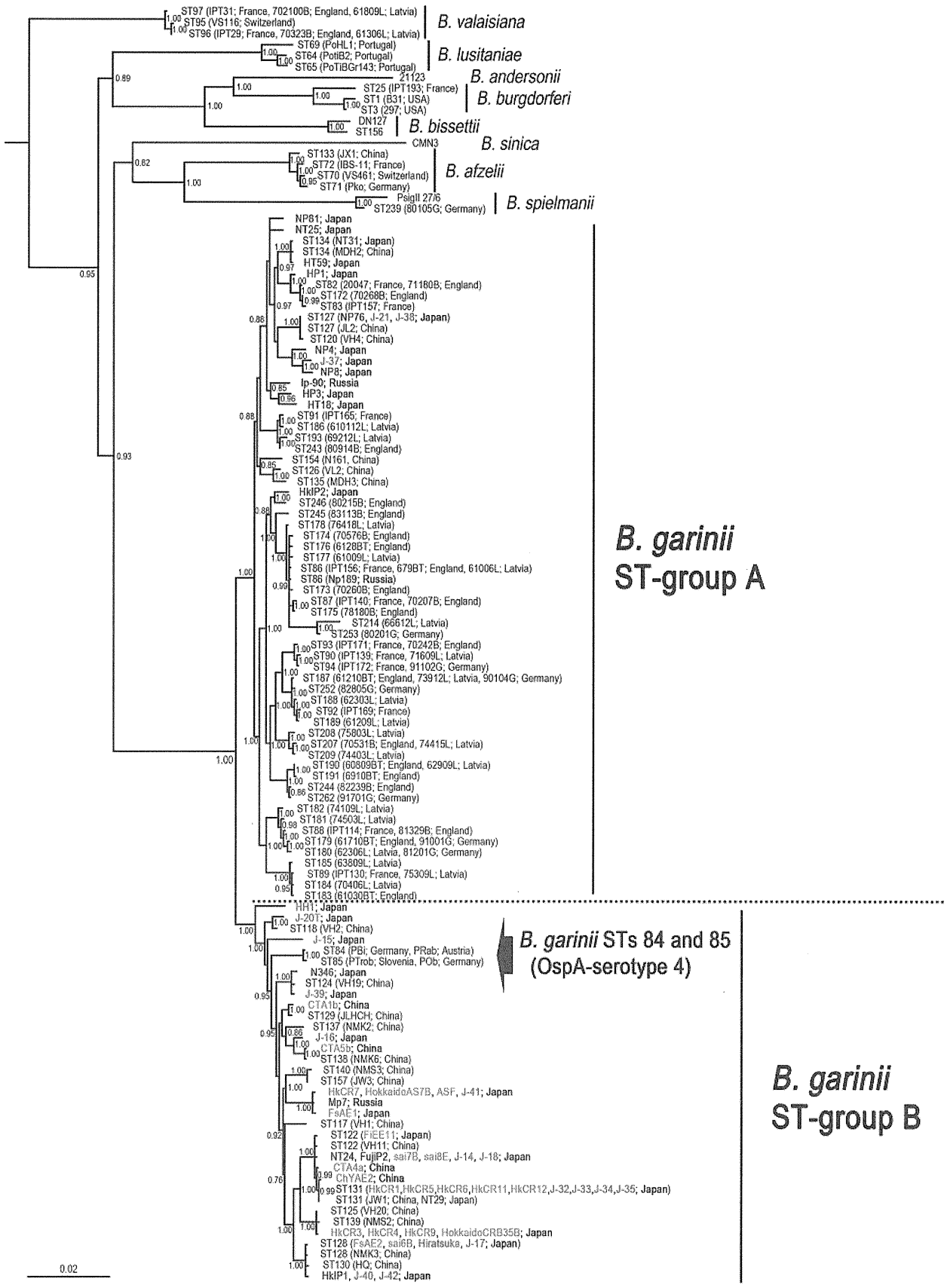
ST97 (IPT31; France, 702100B; England, 61809L; Latvia)
ST95 (VS116; Switzerland)
ST96 (IPT29; France, 70323B; England, 61306L; Latvia)
B. valaisiana
ST69 (PoHL1; Portugal)
ST64 (PotiB2; Portugal)
ST65 (PoTiBGr143; Portugal)
B. lusitaniae
21123
B. andersonii
ST25 (IPT193; France)
ST1 (B31; USA)
ST3 (297; USA)
B. burgdorferi
DN127
ST156
B. bissettii
CMN3
B. sinica
ST133 (JX1; China)
ST72 (IBS-11; France)
ST70 (VS461; Switzerland)
ST71 (Pko; Germany)
B. afzelii
PsigII 27/6
ST239 (80105G; Germany)
B. spielmanii
NP81; Japan
NT25; Japan
ST134 (NT31; Japan)
ST134 (MDH2; China)
HT59; Japan
HP1; Japan
ST82 (20047; France, 71180B; England)
ST172 (70268B; England)
ST83 (IPT157; France)
ST127 (NP76, J-21, J-38; Japan)
ST127 (JL2; China)
ST120 (VH4; China)
NP4; Japan
J-37; Japan
NP8; Japan
Ip-90; Russia
HP3; Japan
HT18; Japan
ST91 (IPT165; France)
ST186 (610112L; Latvia)
ST193 (69212L; Latvia)
ST243 (80914B; England)
ST154 (N161, China)
ST126 (VL2; China)
ST135 (MDH3; China)
HkIP2; Japan
ST246 (80215B; England)
ST245 (83113B; England)
ST178 (76418L; Latvia)
ST174 (70576B; England)
ST176 (6128BT; England)
ST177 (61009L; Latvia)
ST86 (IPT156; France, 679BT; England, 61006L; Latvia)
ST86 (Np189; Russia)
ST173 (70260B; England)
ST87 (IPT140; France, 70207B; England)
ST175 (78180B; England)
ST214 (66612L; Latvia)
ST253 (80201G; Germany)
ST93 (IPT171; France, 70242B; England)
ST90 (IPT139; France, 71609L; Latvia)
ST94 (IPT172; France, 91102G; Germany)
ST187 (61210BT; England, 73912L; Latvia, 90104G; Germany)
ST252 (82805G; Germany)
ST188 (62303L; Latvia)
ST92 (IPT169; France)
ST189 (61209L; Latvia)
ST208 (75803L; Latvia)
ST207 (70531B; England, 74415L; Latvia)
ST209 (74403L; Latvia)
ST190 (60809BT; England, 62909L; Latvia)
ST191 (6910BT; England)
ST244 (82239B; England)
ST262 (91701G; Germany)
ST182 (74109L; Latvia)
ST181 (74503L; Latvia)
ST88 (IPT114; France, 81329B; England)
ST179 (61710BT; England, 91001G; Germany)
ST180 (62306L; Latvia, 81201G; Germany)
ST185 (63809L; Latvia)
ST89 (IPT130; France, 75309L; Latvia)
ST184 (70406L; Latvia)
ST183 (61030BT; England)
B. garinii
ST-group A
HR1; Japan
J-20T; Japan
ST118 (VH2; China)
J-15; Japan
ST84 (PBi; Germany, PRab; Austria)
ST85 (PTrob; Slovenia, POb; Germany)
B. garinii STs 84 and 85
(OspA-serotype 4)
N346; Japan
ST124 (VH19; China)
J-39; Japan
CTA1b; China
ST129 (JLHCH; China)
ST137 (NMK2; China)
J-16; Japan
CTA5b; China
ST138 (NMK6; China)
ST140 (NMS3; China)
ST157 (JW3; China)
HkCR7, HokkaidoAS7B, ASF, J-41; Japan
Mp7; Russia
FsAE1; Japan
ST117 (VH1; China)
ST122 (FiEE1; Japan)
ST122 (VH11; China)
NT24, FujiIP2, sai7B, sai8E, J-14, J-18; Japan
CTA4a; China
ChYAE2; China
ST131 (HkCR1,HkCR5,HkCR6,HkCR11,HkCR12,J-32,J-33,J-34,J-35; Japan)
ST131 (JW1; China, NT29; Japan)
ST125 (VH20; China)
ST139 (NMS2; China)
HkCR3, HkCR4, HkCR9, HokkaidoCRB35B; Japan
ST128 (FsAE2, sai6B, Hiratsuka, J-17; Japan)
ST128 (NMK3; China)
ST130 (HQ; China)
HkIP1, J-40, J-42; Japan
B. garinii
ST-group B
0.02

TABLE 2. ST groups of *Borrelia garinii* isolates from *Ixodes persulcatus* ticks, rodents, and humans

Isolation source	Country	*B. garinii* ST	
		Group A	Group B
I. persulcatus	Japan	HkIP2, HP1, HP3, HT18, HT59, NP4, NP8, NP76, NP81, NT25, NT31	HkIP1, N346, NT24, FujiP2
	Russia	Ip90, Np189	Mp7
Rodents	Japan	None	HkCR1, HkCR3, HkCR4, HkCR5, HkCR6, HkCR7, HkCR9, HkCR11, HkCR12, HokkaidoCRB35B, HokkaidoAS7B, ASF, FsAE1, FsAE2, FiEE11, sai6B, sai7B, sai8E
	China	None	ChYAE2, CTA1b, CTA4a, CTA5b
Humans	Japan	J-21, J-37, J-38	Hiratsuka, J-14, J-15, J-16, J-17, J-18, J-20T, J-32, J-33, J-34, J-35, J-39, J-40, J-41, J-42, HH1

disease in China. In contrast, *B. garinii* ST group A was not isolated from rodents in this study, yet was found to include almost all of the *B. garinii* isolates in Europe. Given that several reports claim *B. garinii* is detectable from birds in Asia (10, 20, 21), we suspect that *B. garinii* ST group A is maintained by birds, as are most of the *B. garinii* isolates in Europe.

I. persulcatus and *I. ricinus* ticks are known vectors of pathogenic *B. garinii* in Asia and Russia and Europe, respectively. In this study, *B. garinii* ST group A, which is most often isolated in Europe, was infrequent in Japan (Fig. 1). The reason remains unclear, but the inhabitant species of ticks may be associated with this geographical difference. Furthermore, Korenberg et al. recently found that *I. pavlovskyi* and *I. persulcatus* ticks differ in their abilities to transmit borrelia (12). This finding may support the notion that the resident tick species contributes to the determination of the endemic species *B. garinii*.

In this study, it was observed that the *B. garinii* strains which infect humans in Japan, are often found in rodents, but not nearly as often in ticks. In Europe, it was reported that *B. garinii* STs 84 and 85 are pathogenic to humans, although they were infrequent among tick isolations (6). These STs are not found in Japan, but they are clustered with most of the Japanese clinical isolates. Therefore, we hypothesized from our data that *B. garinii* ST group B isolates may be more pathogenic to humans than isolates of *B. garinii* ST group A.

In conclusion, *B. garinii* could be divided into two phylogenetic groups by MLST analysis, and one group (*B. garinii* ST group B) was predominant among clinical and rodent isolates in Japan. These results suggest that rodents are the reservoir host for most human-pathogenic *B. garinii* isolates in Japan. We also revealed that Japanese clinical isolates may be distinct from most European isolates. This may be due to the different vectors of *B. garinii* in Asia and Russia versus Europe. Our findings may contribute to the elucidation of *B. garinii*-caused Lyme disease epidemiology.

We thank Gabriele Margos (Department of Biology and Biochemistry, University of Bath) for providing information on MLST; Maki Muto and Yumiko Ogasawara (National Institute of Infectious Disease) for technical assistance; and Bettina Wilske, Kenji Miyamoto, Nanao Sato, Haipeng Wang (China Medical University), and Jichun Wang (China Medical University) for providing *Borrelia* strains.

This study was supported in part by a grant for research on emerging and reemerging infectious diseases from the Japanese Ministry of Health, Labor, and Welfare.

REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
2. **Eisen, L., and R. S. Lane.** 2002. Vectors of *Borrelia burgdorferi* sensu lato, p. 91–115. *In* J. S. Gray, O. Kahl, R. S. Lane, and G. Stanek (ed.), Lyme borreliosis biology, epidemiology and control. CABI Publishing, Oxon, United Kingdom.
3. **Fukunaga, M., M. Sohnaka, Y. Takahashi, M. Nakao, and K. Miyamoto.** 1993. Antigenic and genetic characterization of *Borrelia* species isolated from *Ixodes persulcatus* in Hokkaido, Japan. J. Clin. Microbiol. **31:**1388–1391.
4. **Gern, L.** 2008. *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis: life in the wilds. Parasite **15:**244–247.
5. **Hanincová, K., et al.** 2003. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl. Environ. Microbiol. **69:**2825–2830.
6. **Hu, C. M., B. Wilske, V. Fingerle, Y. Lobet, and L. Gern.** 2001. Transmission of *Borrelia garinii* OspA serotype 4 to BALB/c mice by *Ixodes ricinus* ticks collected in the field. J. Clin. Microbiol. **39:**1169–1171.
7. **Huegli, D., C. M. Hu, P. F. Humair, B. Wilske, and L. Gern.** 2002. *Apodemus* species mice are reservoir hosts of *Borrelia garinii* OspA serotype 4 in Switzerland. J. Clin. Microbiol. **40:**4735–4737.
8. **Ishiguro, F., and N. Takada.** 1996. Lyme *Borrelia* from *Ixodes persulcatus* and small rodents from northern to central parts of mainland Japan. Med. Entomol. Zool. **47:**183–185.
9. **Ishiguro, F., et al.** 1996. Reservoir competence of the vole, *Clethrionomys rufocanus bedfordiae*, for *Borrelia garinii* or *Borrelia afzelii*. Microbiol. Immunol. **40:**67–69.
10. **Ishiguro, F., N. T. Takada, Masuzawa, and T. Fukui.** 2000. Prevalence of Lyme disease *Borrelia* spp. in ticks from migratory birds on the Japanese mainland. Appl. Environ. Microbiol. **66:**982–986.
11. **Kawabata, H., H. Tashibu, K. Yamada, T. Masuzawa, and Y. Yanagihara.** 1994. Polymerase chain reaction analysis of *Borrelia* species isolated in Japan. Microbiol. Immunol. **38:**591–598.
12. **Korenberg, E. I., V. V. Nefedova, V. N. Romanenko, and N. B. Gorelova.** 2010. The tick *Ixodes pavlovskyi* as a host of spirochetes pathogenic for humans and its possible role in the epizootiology and epidemiology of borrelioses. Vector Borne Zoonotic Dis. **10:**453–458.
13. **Li, M., et al.** 1998. Lyme disease *Borrelia* species in northeastern China resemble those isolated from far eastern Russia and Japan. Appl. Environ. Microbiol. **64:**2705–2709.
14. **Margos, G., et al.** 2009. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. Appl. Environ. Microbiol. **75:**5410–5416.
15. **Margos, G., et al.** 2008. MLST of housekeeping genes captures geographic population structure and suggests a European origin of *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. U. S. A. **105:**8730–8735.
16. **Masuzawa, T.** 2004. Terrestrial distribution of the Lyme borreliosis agent *Borrelia burgdorferi* sensu lato in East Asia. Jpn. J. Infect. Dis. **57:**229–235.
17. **Masuzawa, T., et al.** 1996. Comparison of OspA serotypes for *Borrelia burg-*

dorferi sensu lato from Japan, Europe and North America. Microbiol. Immunol. **40:**539–545.
18. **Masuzawa, T., et al.** 2005. Characterization of *Borrelia burgdorferi* sensu lato isolated in Moscow province—a sympatric region for *Ixodes ricinus* and *Ixodes persulcatus*. Int. J. Med. Microbiol. **294:**455–464.
19. **Miyamoto, K., M. Nakao, N. Sato, and M. Mori.** 1991. Isolation of Lyme disease spirochetes from an ixodid tick in Hokkaido, Japan. Acta Trop. **49:**65–68.
20. **Miyamoto, K., Y. Sato, K. Okada, M. Fukunaga, and F. F. Sato.** 1997. Competence of a migratory bird, red-bellied thrush (*Turdus chrysolaus*), as an avian reservoir for the Lyme disease spirochetes in Japan. Acta Trop. **65:**43–51.
21. **Nakao, M., K. Miyamoto, and M. Fukunaga.** 1994. Lyme disease spirochetes in Japan: enzootic transmission cycles in birds, rodents, and *Ixodes persulcatus* ticks. J. Infect. Dis. **170:**878–882.
22. **Nakao, M., K. Miyamoto, N. Kawagishi, Y. Hashimoto, and H. Iizuka.** 1992. Comparison of *Borrelia burgdorferi* isolated from humans and ixodid ticks in Hokkaido, Japan. Microbiol. Immunol. **36:**1189–1193.
23. **Noppa, L., N. Burman, A. Sadziene, A. G. Barbour, and S. Bergström.** 1995. Expression of the flagellin gene in *Borrelia* is controlled by an alternative sigma factor. Microbiology **141:**85–93.
24. **Piesman, J., and L. Gern.** 2008. Lyme borreliosis in Europe and North America, p. 220–252. *In* A. S. Bowman and P. A. Nuttall (ed.), Ticks: biology, disease and control. Cambridge University Press, Cambridge, United Kingdom.
25. **Takada, N., et al.** 2001. Lyme disease *Borrelia* spp. in ticks and rodents from northwestern China. Appl. Environ. Microbiol. **67:**5161–5165.
26. **Taragel'ová, V., et al.** 2008. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. Appl. Environ. Microbiol. **74:**1289–1293.
27. **Wilske, B., et al.** 1993. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J. Clin. Microbiol. **31:**340–350.
28. **Yamaguti, N., V. J. Tipton, H. L. Keegan, and S. Toshioka.** 1971. Ticks of Japan, Korea, and the Ryukyu Islands. Brigham Young Univ. Sci. Bull. Biol. Ser. **15:**142–148.
29. **Yanagihara, Y., and T. Masuzawa.** 1997. Lyme disease (Lyme borreliosis). FEMS Immunol. Med. Microbiol. **18:**249–261.

Journal of Medical Microbiology (2012), 61, 266–273 DOI 10.1099/jmm.0.034868-0

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

Chihiro Katsukawa,[1] Takako Komiya,[2] Hiroaki Yamagishi,[3] Atsushi Ishii,[3] Shunji Nishino,[3] Shinya Nagahama,[4] Masaaki Iwaki,[2] Akihiko Yamamoto[2] and Motohide Takahashi[2]

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

[1]Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka, Japan
[2]Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan
[3]Osaka Prefectural Dog Management Office, Osaka, Japan
[4]Animal Protection and Livestock Division, Department of Environment, Agriculture, Forestry and Fisheries, Osaka Prefectural Government, Osaka, Japan

Received 16 June 2011
Accepted 14 September 2011

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

INTRODUCTION

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

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

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

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

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

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

METHODS

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

034868 © 2012 SGM *Printed in Great Britain*

sample collection was usually carried out twice a week, on Tuesdays and Fridays. Throat swabs were collected immediately after euthanasia and stored in preservation medium (SEEDSWABγ3 '*Eiken*'; Eiken Chemical) at 4 °C.

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

Toxigenicity testing and nucleotide sequencing of the *tox* gene. Toxigenicity testing was performed by modified Elek test (Engler *et al.*, 1997; Katsukawa *et al.*, 2009; Reinhardt *et al.*, 1998), Vero cell cytotoxicity and neutralization tests (Katsukawa *et al.*, 2009; Miyamura *et al.*, 1974) and PCR for the diphtheria toxin (*tox*) gene. Primers used for PCR are listed in Supplementary Table S1 in JMM Online. Primers Tox 1 and Tox 2 (Nakao & Popovic, 1997), corresponding to the A subunit of the toxin, were first used for detection of the *tox* gene. In isolates negative for the primer pair, further PCR analyses using primers Dipht 6F and Dipht 6R (Nakao *et al.*, 1996) for the B subunit and toxFw and toxRv for the entire *tox* gene (Seto *et al.*, 2008) were carried out to confirm the absence of the gene. The nucleotide sequence of the *tox* gene was determined by amplifying the entire gene fragment with primers toxFw and toxRv (Seto *et al.*, 2008) (Supplementary Table S1) and then by sequencing the fragment with primers placed at appropriate intervals. A phylogenetic tree of *tox* nucleotide sequences was constructed using the neighbour-joining method (Saitou & Nei, 1987).

Table 1. Bacterial strains and isolates used in this study

Strains used for comparison studies are listed here.

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

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

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

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

RESULTS

Isolation of *C. ulcerans* and toxigenicity

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

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

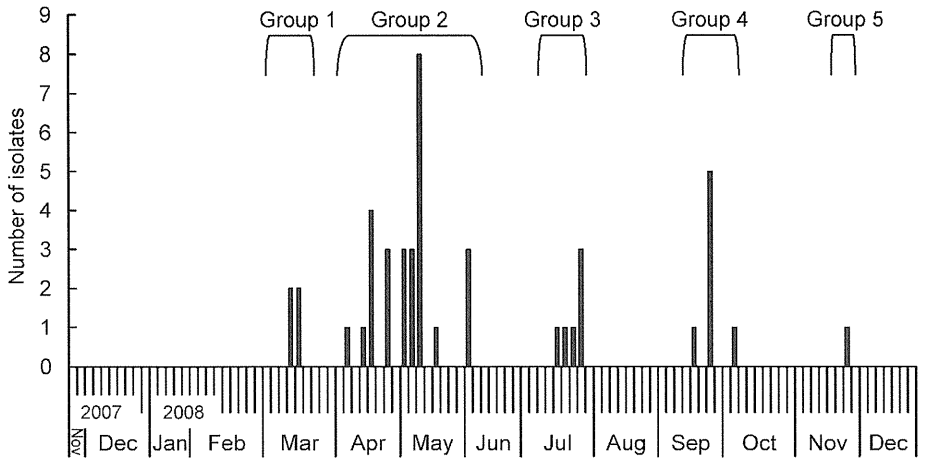

Fig. 1. Chronological representation of *C. ulcerans* isolation. Samples were collected as described in the text. Each sampling period is expressed as a small cell between the horizontal axis and month indications. Bars represent the number of isolates obtained during each sampling period.

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

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

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

PFGE analysis

All 45 isolates and the previous dog and human isolates were compared using PFGE. The isolates were classified into four types (A2, B, C, D) by PFGE (Table 2 and Fig. 2). PFGE classifications of isolates were as follows: PFGE type C included group 1 isolates (four toxigenic strains isolated in March); type A2 included groups 2, 3, 4-1 and 4-2 (37 toxigenic strains isolated from April to October and two of three non-toxigenic strains isolated in September); type B included group 4-3 (one non-toxigenic strain isolated in September); and type D included group 5 (one toxigenic strain isolated in November). As shown in Fig. 2, subtype A2, together with two other subtypes classified according to slight differences in PFGE patterns, composed type A. Previously isolated human strains have been classified with respect to PFGE type as follows: PFGE type A1, human isolates 0102 and 0210 (Komiya *et al.*, 2010) that were closely related to type A2, including 0509 (Asakura *et al.*, 2006) and 0902 (Noguchi *et al.*, 2009), and type A3 including 0607 (Hagiwara *et al.*, 2006). Another human isolate, 0510 (Nureki *et al.*, 2007), was classified in a comparatively distant type D.

Analysis of the diphtheria toxin (*tox*) gene

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

Table 2. Characteristics of the isolates

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

*Month/day in 2008.
†M, Male; F, female.
‡BA, Blood agar; C, charcoal–tellurite blood agar.
§CLDM, Clindamycin; LVFX, levofloxacin; R, resistant; I, intermediate.
‖Isolated from the same dog.

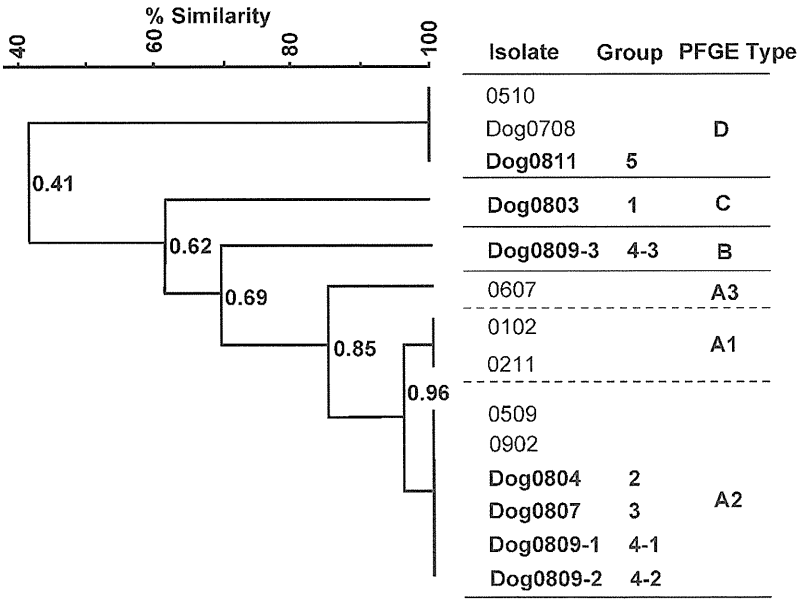

Fig. 2. Phylogenetic analysis of *C. ulcerans* isolates. *C. ulcerans* dog isolates obtained in this study (indicated in bold) as well as Japanese human isolates reported previously (Asakura *et al.*, 2006; Hagiwara *et al.*, 2006; Hatanaka *et al.*, 2003; Komiya *et al.*, 2010; Noguchi *et al.*, 2009;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

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

Antibiotic sensitivity

None of the strains were resistant to benzylpenicillin, ampicillin, cefazolin, cefotiam, cefotaxime, cefaclor, cefditoren, flomoxef, imipenem, meropenem, erythromycin, minocycline, vancomycin or sulfamethoxazole–trimethoprim. The CLDM MIC was $\geqslant 2$ 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

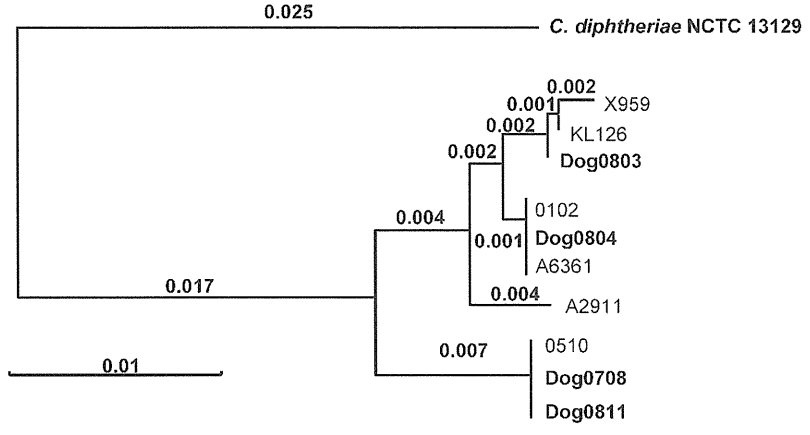

Fig. 3. Phylogenetic analysis based on nucleotide sequences of the *tox* genes. The *tox* gene sequences for the *C. ulcerans* isolates obtained in the present study were determined and compared to the previously published *tox* gene sequences of *C. ulcerans* strains isolated from humans and the *C.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

ACKNOWLEDGEMENTS

This work was supported in part by Health and Labour Sciences Research Grants (Research on Emerging and Re-emerging Infectious Diseases and Research on Pharmaceutical and Medical Safety, H19-Shinkou-Ippan-009).

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

A. Hotta[1], K. Tanabayashi[1], Y. Yamamoto[1], O. Fujita[1], A. Uda[1], T. Mizoguchi[2] and A. Yamada[1]

[1] Department of Veterinary Science, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan
[2] Fukushima Wildlife Rehabilitation Center, Otama, Fukushima, Japan

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

Correspondence:
Akitoyo Hotta, DVM, PhD. Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel.: +81 3 5285 1111; Fax: +81 3 5285 1179;
E-mail: ahotta@nih.go.jp

Received for publication November 30, 2010

doi: 10.1111/j.1863-2378.2011.01422.x

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).

© 2011 Blackwell Verlag GmbH

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.

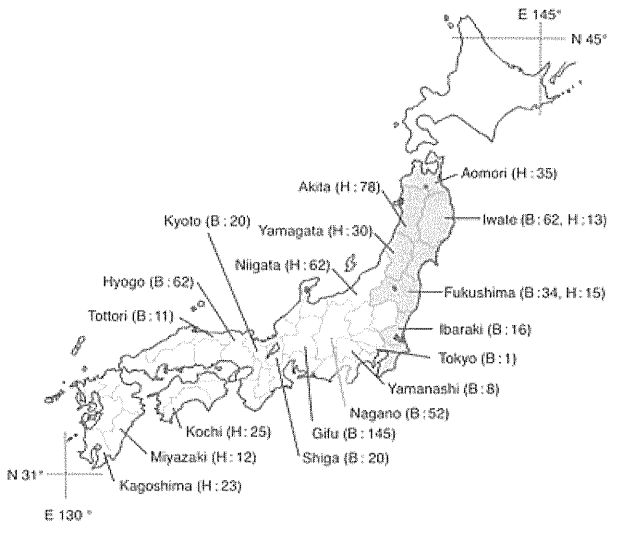

Fig. 1. Map of Japan showing the areas where samples were collected. The numbers of samples collected in each area was shown in parentheses (B, Japanese black bears; H, Japanese hares). The areas coloured grey are the prefectures where more than 50 cases of human tularemia have been reported previously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

© 2011 Blackwell Verlag GmbH

(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

© 2011 Blackwell Verlag GmbH