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.

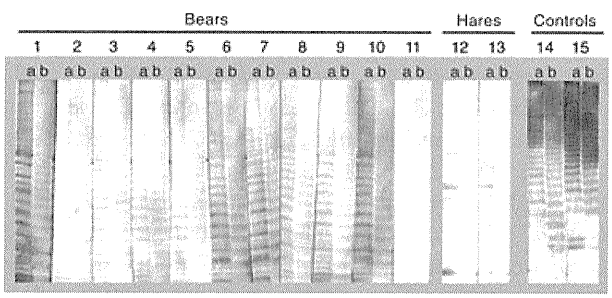

Fig. 2. Detection of antibodies to *Francisella tularensis* antigen by western blot. Sera from 10 black bears (sheets 1–10) reacted specifically with whole cell lysate (a) and purified LPS (b). Thirteen other samples tested positive in the screening MA did not show any positive reaction with either antigens 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.

Acknowledgements

The authors are grateful to Dr Mikiko Aoki (Iwate University, Iwate), Prof. Toshio Tsubota (Hokkaido University), Dr Atsushi Katayama (Wildlife Management Office, Hyogo), Dr Sachiko Nakamura (University of Hyogo, Hyogo) and Ms Rumiko Nakashita (Tokyo Metropolitan University, Tokyo) for providing samples from Japanese black bears. The authors also thank the volunteer hunters and Dai-Nippon Ryo-Yu-Kai (Japanese Hunters association) for hunting Japanese hares and providing the samples. This study was supported by the Health and Labour Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare in Japan.

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.

Jpn. J. Infect. Dis., 64, 345-348, 2011

Epidemiological Report

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

Akiko Okutani[1†], Hurelsukh Tungalag[2†], Bazartseren Boldbaatar[1,3], Akio Yamada[1], Damdindorj Tserennorov[2], Ishtsog Otgonchimeg[2], Adiya Erdenebat[2], Dashdavaa Otgonbaatar[2], and Satoshi Inoue[1*]

[1]*Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo 162-8640, Japan;*
[2]*National Center for Infectious Diseases with Natural Foci, Ulaanbaatar, Mongolia; and*
[3]*School of Veterinary Medicine, Nihon University, Kanagawa 252-8510, Japan*

(Received April 20, 2011. Accepted June 13, 2011)

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

*Corresponding author: Mailing address: Department of Veterinary Science, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81 3 5285 1111, Fax: +81 3 5285 1179, E-mail: sinoue@nih.go.jp

†These authors contributed equally to this study.

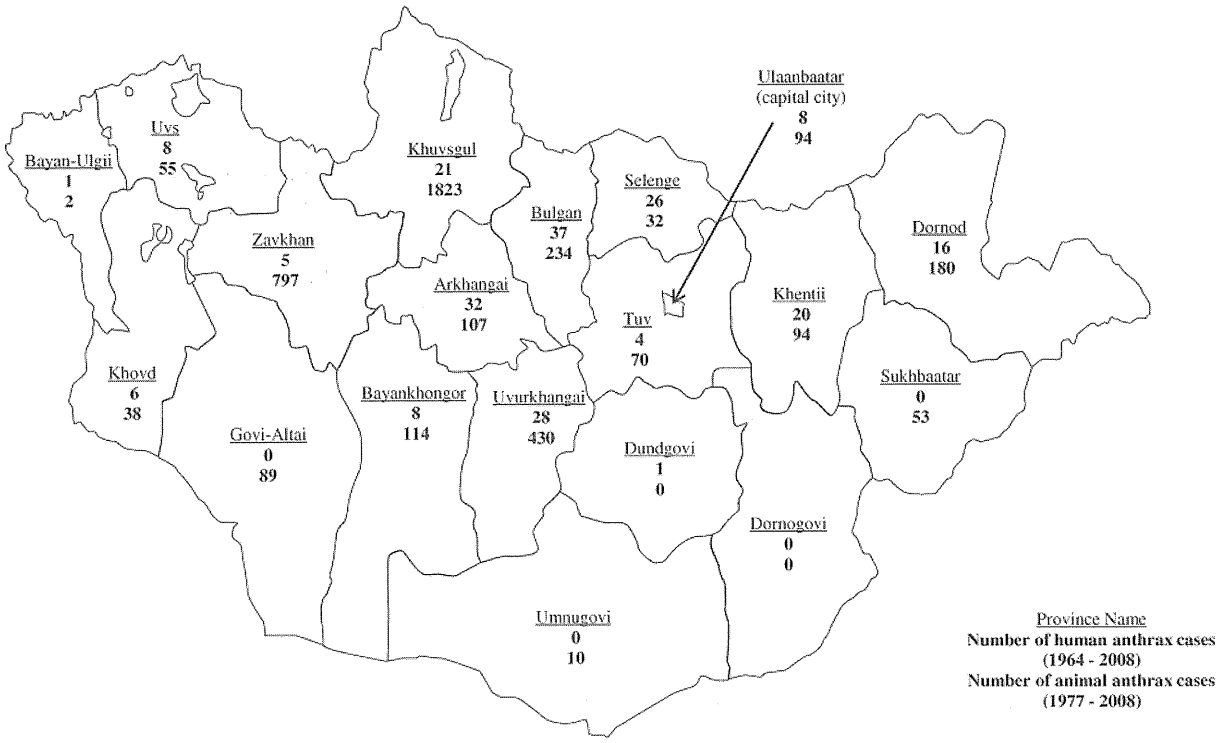

Fig. 1. Provinces of Mongolia with accumulative number of human anthrax cases of each province from 1964 to 2008 (upper column) and livestock from 1977 to 2008 (lower column) are shown.

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

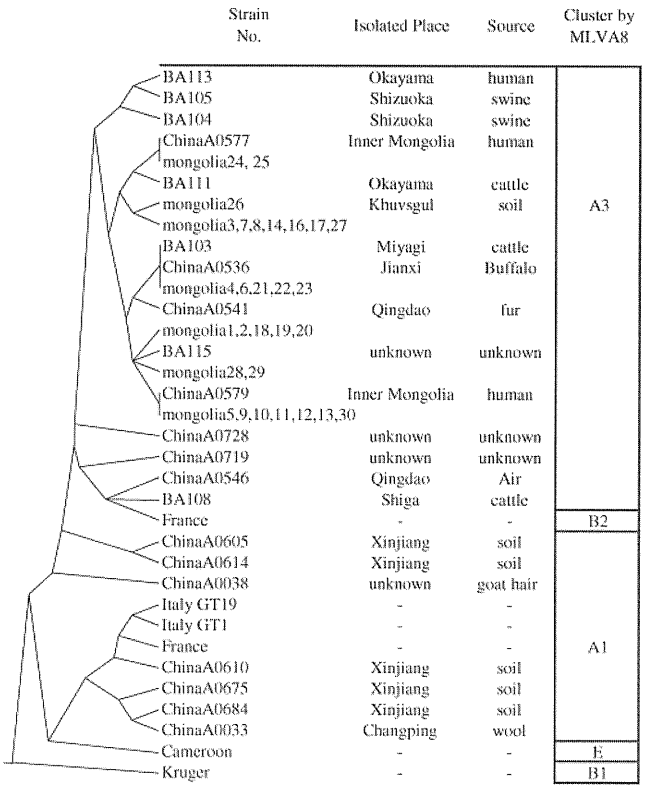

Fig. 2. Dendrogram constructed according to the MLVA-8 of *B. anthracis* strains including Mongolian isolates. The tree was drawn using UPGMA method as previously reported (3).

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.

Acknowledgments The authors gratefully acknowledge to the technical support and discussion of Dr. Tsuyoshi Sekizuka and Dr. Makoto Kuroda (NIID, Japan) for the analysis of sequencing and dendrogram.

This study was supported by the Health and Labour Science Research grant from the Ministry of Health, Labour and Welfare of Japan.

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.