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

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

Materials and Methods

Blood samples

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

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

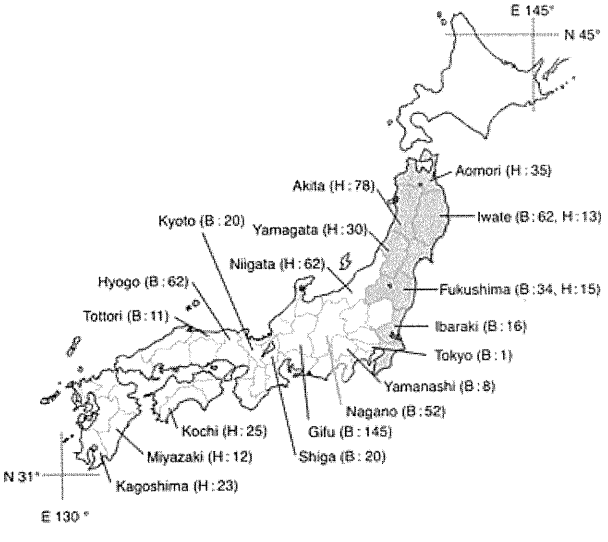

Fig. 1. Map of Japan showing the areas where samples were collected. The numbers of samples collected in each area was shown in parentheses (B, Japanese black bears; H, Japanese hares). The areas coloured grey are the prefectures where more than 50 cases of human tularemia have been reported previously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

Bacterial antigens

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

Micro-agglutination test (MA)

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

Enzyme-linked immunosorbent assay (ELISA)

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

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

SDS-PAGE and western blotting (WB)

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

Indirect immunofluorescence assay

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

Criterion of positive reaction

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

Cross-reactivity with other bacterial antigen

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

Results

Screening assays

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

Confirmatory assays

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

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

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

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

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

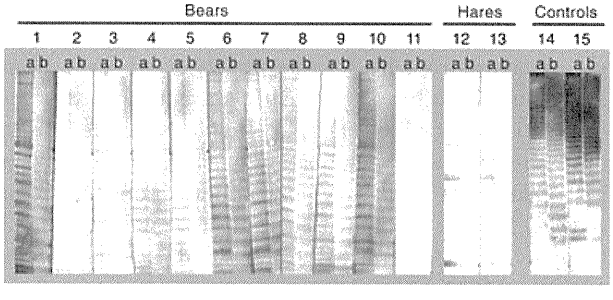

Fig. 2. Detection of antibodies to *Francisella tularensis* antigen by western blot. Sera from 10 black bears (sheets 1–10) reacted specifically with whole cell lysate (a) and purified LPS (b). Thirteen other samples tested positive in the screening MA did not show any positive reaction with either antigens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

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

Discussion

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

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

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

The blanks indicate no sample or 0%.

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

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

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

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

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

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

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

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