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Short Communication

Survey of *Francisella tularensis* in Wild Animals in Japan in Areas Where Tularemia is Endemic

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SUMMARY: Samples taken from 428 wild animals and 126 ticks, collected from a tularemia-endemic area in Japan between 2005 and 2013, were analyzed for the presence of *Francisella tularensis*. *F. tularensis* was isolated from a Japanese hare carcass whereas the samples from live animals and ticks were negative for *F. tularensis* by real-time PCR. Our results suggest that *F. tularensis* is still present in Japan although its prevalence is considerably low even in areas where tularemia is endemic.

Francisella tularensis is the etiological agent of the zoonotic disease tularemia. It has a very wide host range including mammals, birds, amphibians, fish, and invertebrates (1), and is able to remain infectious in water and mud for months (2). Tularemia is endemic in many regions of the Northern hemisphere, and in Europe, it has reemerged in several countries including Germany (3), Kosovo (4), and Turkey (5). In these countries, certain rodent species and lagomorphs are of paramount importance for maintaining enzootic foci (6) and a high rodent population is thought to trigger the outbreaks in humans (4). In Japan, tularemia is endemic in Tohoku district, the northeastern area of the largest-island, Honshu, and approximately 1,400 cases of human tularemia have been reported since 1924 (7). *F. tularensis* has been isolated from human patients, Japanese hares, a Japanese shrew-mole, and ticks (8). Four of the 5 patients diagnosed with tularemia in 2008 acquired the infection from Japanese hares (9). Although several other animal species have also been implicated as the source of infection (7), the epizootic transmission cycle of *F. tularensis* is yet to be understood. Our previous studies showed that wild animals that tested positive for antibodies to *F. tularensis* were exclusively found within an area in Japan where tularemia is endemic (10,11). In order to better understand how this zoonotic pathogen is maintained in nature, identification of wild animals harboring infectious bacteria is necessary. In this study, we attempted to determine the prevalence of *F. tularensis* in wild animals and ticks, through the detection of *F. tularensis*-specific nucleic acid, in an area of Japan where tularemia is endemic.

Samples were collected in Nikaho city (Akita prefecture), Namie town (Fukushima prefecture), and Yokohama town (Aomori prefecture) (Fig. 1). The former 2 are located in the major areas where tularemia has been endemic historically (7), and the latter lies within a 1.5-km radius from the point where an *F. tularensis* infected Japanese hare was found in May 2008 (12). In Akita, 50 Japanese hares (*Lepus brachyurus*) were shot by volunteer hunters during the hunting season in 2006, 2008, and 2009 (Table 1). The livers and/or spleens of hares were sampled and subsequently sent to the National Institute of Infectious Diseases (NIID) under refrigeration. A Japanese hare incidentally found dead by a volunteer hunter was also sent to the NIID in accordance with the safe transport guidelines stipulated by the institute. A total of 181 spleens from 103 large Japanese field mice (*Apodemus speciosus*) and 78 small Japanese field mice (*Apodemus argenteus*) collected in Fukushima prefecture between 2012 and 2013 (Table 1) were kindly provided by Dr. Onuma (National Institute of Environment, Tsukuba, Ibaraki, Japan). In Aomori prefecture, Sherman traps were set in November 2008, June 2009, and June 2010. A total of 196 small mammals, consisting of 80 large Japanese field mice, 34 small Japanese field mice, 63 Japanese grass voles (*Microtus montebelli*), 17 Japanese shrew-moles (*Urotrichus talpoides*), and 2 shinto shrews (*Sorex shinto*) were captured (Table 1). These small mammals were humanely euthanized and the spleens and livers were removed.

A total of 126 ticks were collected from 17 Japanese hares hunted in Akita prefecture and 19 small mammals captured in Aomori prefecture (Table 1). The majority of the ticks collected from Japanese hares were nymphs, predominantly *Haemaphysalis flava* and *Ixodes ovatus*,

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Table 1. Number of wild animal and tick for molecular detection of *Francisella tularensis*

Prefecture	Yr	Sampling Period	Japanese hare	Small mammal					Total of wild animals	Ticks	Total
				Large Japanese field mouse	Small Japanese field mouse	Japanese grass vole	Japanese shrew-mole	Shinto shrew			
Akita	2006	Mar.	4						4	0	4
	2008	Mar. and Nov.–Dec.	25						25	100	125
	2009	Jan.–Mar.	21 (1)[1)]						21 (1)[1)]	0	21 (1)[1)]
Aomori	2008	Nov.		13	9	31	1	1	55	7	62
	2009	June		16	11	22	5	0	54	4	58
	2010	June		51	14	10	11	1	87	15	102
Fukushima	2012	Aug.–Nov.		67	74				141	0	141
	2013	July–Oct.		36	4				40	0	40
Total			50 (1)[1)]	183	112	63	17	2	427 (1)[1)]	126	553 (1)[1)]

[1)]: Number in parenthesis indicate carcass sample.

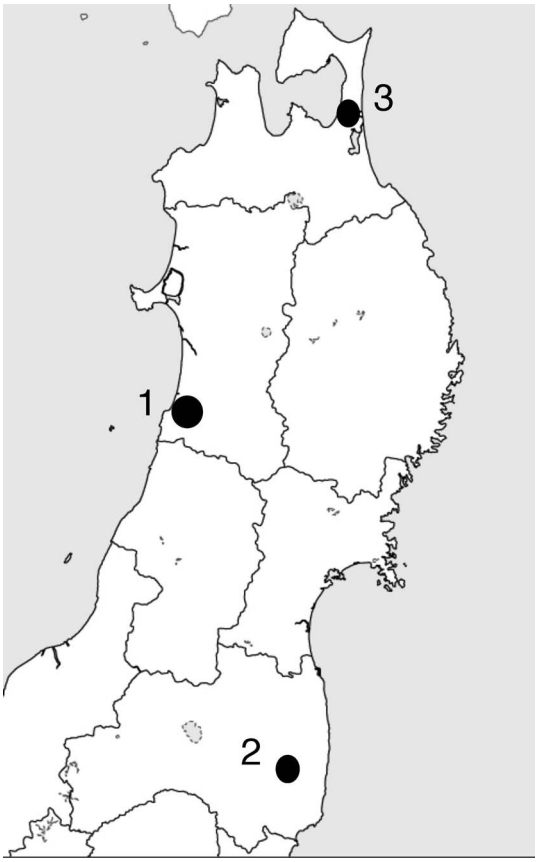

Fig. 1. Surveillance areas in Tohoku district in Japan. The location of sampling areas, Nikaho city in Akita prefecture, Namie town in Fukushima prefecture, and Yokohama town, Aomori prefecture were numbered 1, 2, and 3, respectively.

whilst those collected from small mammals were predominantly nymphs of *I. ovatus* and *Ixodes monospinosus*. For the extraction of nucleic acid, nymphs and larvae, with the exclusion of overtly engorged ticks, were separated into 18 pools (with 2 to 6 ticks per pool) according to the individual host on which they were found.

Nucleic acid was extracted from the spleen or liver of wild animals and tick samples by using the DNeasy Blood & Tissue (Qiagen, Hilden, Germany) or NucleoSpin® tissue (Macherey-Nagel Inc., Bethlem, UK), according to the manufacturer's instructions. The concentration and purity of the extracted nucleic acid samples were determined with Nanodrop ND-3000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and were stored at −30°C until analysis. The samples were subsequently analyzed for the presence of *F. tularensis* DNA by real-time TaqMan PCR, with primers targeting the *tul4* gene (13), and real-time PCR with primers targeting the *fopA* gene and hybridization probes (14). All the PCRs were carried out on the Light Cycler System (Roche Diagnostics, Mannheim, Germany). Every PCR run included purified water as a negative control and DNA of *F. tularensis* Schu strain as a positive control. The samples with positive reactions in both of the real-time PCRs were considered *F. tularensis*-positive. All of the samples from wild animals captured in Aomori and Akita prefectures were also subjected for the isolation of bacteria as previously described (12).

F. tularensis DNA was not detected in the samples from the 427 healthy wild animals or from the 126 ticks. Most of the small mammals captured in Aomori prefecture carried ectoparasites other than ticks, such as mites or fleas, but none of them carried *F. tularensis* DNA (data not shown). The samples from this area were obtained from 3 seropositive rodents (2 Japanese grass voles and a large Japanese field mouse) as previously reported (11). *F. tularensis* DNA was not detected in the sero-positive rodents, and was only detected in the spleen and liver of a Japanese hare carcass found in Akita prefecture in 2009 (Table 1). The carcass was normal in appearance; however, at necropsy, marked splenomegaly was observed (Fig. 2A) with multiple white foci/lesions in its section (Fig. 2B). From 1 milligram of splenic tissue of this carcass, approximately 6.1×10^7 copies of *fopA* gene and 9.7×10^{10} copies of *tul4* gene were detected. This discrepancy could be attributed to the fact that the *tul4* gene may be more amplifiable than the *fopA* gene, as discussed by Higgins et al. (15). The isolated bacterium, which formed tiny gray-white colonies on chocolatized Eugon agar (Fig. 2C), was Gram-negative (Fig. 2D); however, the reaction of a monoclonal antibody specific to *F. tularensis*

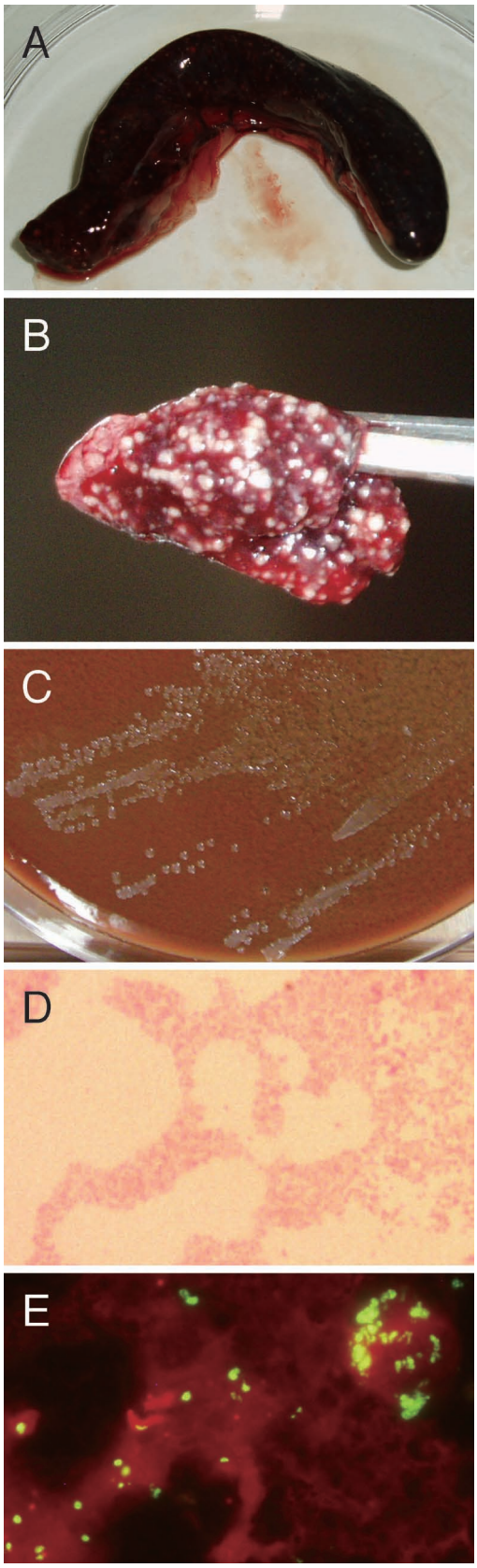

Fig. 2. (Color online) Pathological finding on the spleen of Japanese hare carcass and identification of bacteria in the PCR-positive sample. At necropsy, marked enlargement of the spleen (A) and multiple white foci at the section (B) were observed. The isolated bacterium, that formed tiny white-gray colony on chocolatized Eugon agar plate (C), was negative in Gram-stain (D). Monoclonal antibody specific to *F. tularensis* lipopolysaccharide reacted with the bacterial smear on an impression of lung (E).

lipopolysaccharide (16) with smears of the bacteria on impressions of the spleen, liver, lung (Fig. 2E), and kidney allowed its identification as *F. tularensis*. This isolate was termed NVF1 and was found to be *F. tularensis* subsp. *holarctica* biovar *japonica* by molecular techniques as previously reported (9). Bacterial burdens in the spleen, lung, liver, and kidney were 6×10^4, 10^4, 10^5, and 50 cfu/mg, respectively.

In this study, the positive rate of *F. tularensis* DNA among wild animals was 0.2% (1/428). In European countries where tularemia is endemic, several epidemiological surveys in rodents have been conducted. In Germany, the *F. tularensis* DNA positive rate among small mammals was 4.9% (3). In Ukraine, *F. tularensis* were isolated from 20.1% of the samples from mammals notably the samples obtained from rodents of *Microtus* spp. (4.8%) (17). In Kosovo, *F. tularensis* antigen positive rates among mice and hares were reported to be 10.2% (18). The prevalence of *F. tularensis* among wild animals in the areas where tularemia is endemic is likely to be lower in Japan than in Europe. On the other hand, the prevalence of *F. tularensis*-positive ticks varied among the reports, even within Europe (3,19). As an annual change in tick population on Japanese hares was observed (Table 1), further research on ticks sucking on Japanese hares may be necessary to improve our current understanding of the ecology of *F. tularensis* in Japan.

The fact that infectious bacteria were isolated from a Japanese hare carcass indicates that *F. tularensis* continues to exist in Akita prefecture despite not having been detected since 1997, the year when the latest human case in Akita prefecture was reported (20). Bacterial burdens in the organs of the carcass were lower than those obtained from experimentally infected mice (21). This may be attributed to tissue degradation, as the carcass was left for a long time after the animal's death. The Japanese hare is a nocturnal herbivore, residing solely in a simple shelter with a home range that is much wider than those of rabbits or small rodents (22). The incubation period of tularemia in hares would be 1 to 10 days (23). Thus, it is difficult to elucidate when and where this Japanese hare was infected with *F. tularensis*. Our unpublished data showed that 10^8 colony forming units of *F. tularensis* did not survive at 20°C for 100 days. Genchi et al. reported that *F. tularensis* did not transmit vertically in ticks (24). Considering these facts together, it is imperative to conduct immediate epidemiological research when Japanese hare carcasses are found in a region where tularemia is endemic, to aid in establishing the lifecycle of *F. tularensis* and identifying the source of the infection.

The sampling area in Fukushima prefecture is located in the Abukuma mountains where a number of human tularemia cases have been reported (25). The sampling area has been designated as the exclusion zone as of March 2011 due to the Fukushima nuclear power plant accident. All human residents in this area have been forced to evacuate, and animals have subsequently reclaimed the living range of humans and changed both their behavioral patterns and habits (26). Thus, the prevalence and lifecycle of *F. tularensis* in the area might have changed in the future. In this regard, continuous monitoring will provide invaluable data to evaluate the effects of unmanned settlements, non-

environmental protection, and radiation on wildlife, as well as of the pathogenesis of zoonotic diseases.

Considering the current situation, molecular and bacterial surveys among small healthy mammals and ticks will be a cost-ineffective method for understanding tularemia ecology even in those areas of Japan where tularemia has been endemic. In European countries, climate change (5) and the population of rodents (4) are thought to profoundly influence the rate of tularemia occurrence. Targeted surveys on indicator animals like the Japanese hare, carcasses in particular, and the tick population on wild animals may be important to detect rare zoonotic diseases such as tularemia. To achieve this, interdisciplinary or trans-disciplinary collaborative efforts underpinned by the One Health concept would be invaluable.

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Conflict of interest None to declare.

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