Francisella tularensis is the etiologic 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 infections 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.

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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.
whilst those collected from small mammals were predominantly nymphs of *I. ovatus* and *Ixodes monospinosis*. 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 $6.1 \times 10^7$ copies of *fopA* gene and $9.7 \times 10^{10}$ 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 *Francisella tularensis* lipopolysaccharide reacted with the bacterial smear on an impression of lung (E).

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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