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Short Communication

Identification of the Source of *Francisella tularensis* Infection by Multiple-Locus Variable-Number Tandem Repeat Analysis

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SUMMARY: Tularemia is a zoonotic disease caused by *Francisella tularensis*. Most patients in Japan have reportedly acquired such infections through direct contact with infected Japanese hares. We recently encountered a patient who contracted tularemia after skinning and butchering a dead hare. Because the remains of the hare were available, we attempted to determine whether the patient actually contracted infection by handling the carcass. *F. tularensis*-specific sequences were successfully amplified by PCR from the patient specimens as well as from the remnants of discarded hare carcass. PCR amplification of the IS*Ftu2* and RD1 regions indicated infection by *F. tularensis* subsp. *holarctica*, which was considered as a prevalent strain in Japan. Furthermore, high-resolution multiple-locus variable-number tandem repeat analysis (MLVA) showed that the combination of repeat numbers in sequences from the patient and hare were indistinguishable, thus indicating that the patient had been infected with *F. tularensis* strain that had also infected the hare. These findings demonstrated that MLVA is a useful epidemiological investigational tool to identify possible sources of certain zoonotic diseases such as tularemia.

Francisella tularensis is a highly pathogenic gram-negative coccobacillus identified as the causative agent of zoonotic tularemia in small mammals and humans. *F. tularensis* comprises four subspecies: *tularensis*, *holarctica*, *novicida*, and *mediasiatica*. These are characterized by differences in biochemical properties, virulence, and geographical distribution (1). *F. tularensis* subsp. *tularensis* is considered as a potential bioterrorism agent (1,2). Among the four subspecies, only *holarctica* has been confirmed in Japan. These bacteria are primarily maintained in lagomorphs and rodents as host animals and transmitted to humans through various routes, including the handling of infected or dead animals; tick or insect bites; contact with or ingestion of infected water, food, or soil; and inhalation of infective aerosols (1). In Japan, from 1924 to 1994, 1,372 human cases of tularemia were diagnosed, and most of these patients were shown to have acquired the disease through direct contact with infected hares or their tissues (3). Thus, tularemia cases are very rarely encountered in Japan.

High-resolution multiple-locus variable-number tandem repeat analysis (MLVA) was developed for the molecular typing of several bacterial species, including *Francisella* spp. (4–8). It has been shown to be highly effective in the discrimination of individual strains. We recently performed MLVA of 33 Japanese *Francisella* strains and found that the genomic diversity was higher in Japanese strains than in those from other countries (5).

In March 2008, a case of serologically confirmed tularemia was reported in Fukushima Prefecture, Japan. Because the patient had a history of handling the carcass of a Japanese hare and because the remains of the dead animal were available, we attempted to determine the genetic diversity between the *F. tularensis* identified in the patient and that identified in dead hare samples via MLVA.

After skinning and butchering the carcass of a Japanese hare (*Lepus brachyurus angustidens*) without gloves, a 57-year-old male hunter presented with fever and an enlarged right axillary lymph node, and a serological diagnosis of tularemia was made at the Ohara Research Laboratory, Ohara General Hospital, Fukushima, Japan. Pus samples were aspirated from the enlarged lymph node of the patient 44 days after disease onset (9). The remaining hare carcass was recovered from a plastic trash box placed outside the patient's house 65 days after skinning and butchering the animal, and the decomposed muscles were subjected to DNA extraction.

Bacterial strains of *F. tularensis* subsp. *tularensis* Schu and *F. tularensis* subsp. *holarctica* Yama and live vaccine strain (LVS) were used in this study. The KU-1 strain isolated from a dead Japanese hare (*L. brachyurus angustidens*) in Aomori Prefecture in 2008 (10) was

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kindly provided by Dr. Hisaaki Sato, Kitasato University (Aomori, Japan). The NVF1 strain isolated in our laboratory was from a dead Japanese hare (*L. brachyurus angustidens*) found in Akita Prefecture in 2008.

DNA extraction from the samples was performed using the DNeasy Blood & Tissue® Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA samples from the patient and the hare were extracted in a different safety cabinet on different days to prevent cross-contamination. The culture conditions for KU-1 and NVF1 strains and the preparation methods of the bacterial DNA have been described previously (5). The extracted DNA was stored at −30°C until analyzed by PCR.

First, all samples were subjected to PCR amplification with primer sets specific for the 16S rRNA, *fopA*, and *tul4* genes according to previously described protocols (11–13). The PCR products were then purified using the NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Nagel, Düren, Germany). Direct sequencing was performed by using the Big Dye Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). For identification at the subspecies or strain level, a conventional PCR protocol employing primers targeting IS*Ftu2* and the RD1 regions of *F. tularensis* was used as described previously (14,15).

We determined the number of tandem repeats located at seven variable-number tandem repeat (VNTR) loci (Ft-M2, Ft-M3, Ft-M6, Ft-M10, Ft-M18, Ft-M20, and Ft-M25), as described previously (5).

Subsequently, we attempted to isolate bacteria by cultivating either the patient pus or hare muscle samples on Eugon chocolate agar plates (16); however, no bacteria were isolated. Therefore, *F. tularensis* genomic DNA was amplified by PCR that targeted the 16S rRNA, *fopA*, and *tul4* genes, and all PCR products were directly sequenced. All gene sequences from the patient and hare samples showed 100% identity to those from *F. tularensis* subsp. *holarctica* LVS (Table 1) and Schu (data not shown), thus indicating that both the patient and hare were infected with *F. tularensis*.

We further conducted PCR targeting of the IS*Ftu2* or RD1 regions to identify the *F. tularensis* subspecies. The DNA amplicons (1,249 bp) were amplified from the patient and hare samples using IS*Ftu2*-PCR. The amplicon sizes were identical to those of type B *F. tularensis* LVS (Fig. 1, lanes 1, 2, and 3) but different from those of type A *F. tularensis* Schu strain (Fig. 1, lane 4). The results of RD1-PCR indicated that the size of the amplified DNA fragments (1,135 bp) obtained from the patient and hare samples were exactly the same as that of the *F. tularensis* Yama strain, a representative Japanese strain (Fig. 1, lanes 5, 6, and 7); however, the size of the amplicon was larger than that obtained from the *F. tularensis* LVS strain (924 bp, Fig. 1, lane 8). These results collectively indicated that the *F. tularensis* strains identified in the patient and hare belonged to the Japanese lineage of *F. tularensis* subsp. *holarctica*.

To better elucidate the causative relationship between the *F. tularensis* sequences detected in our human

Table 1. Identity of *Francisella*-specific gene sequences

	Sequence identity (%)[1]			
	Gene (amplicon size)	16S rRNA (1,011 bp)[2]	*fopA* (707 bp)[3]	*tul4* (428 bp)[4]
DNA samples				
Hare		100	100	100
Patient		100	100	100

[1]: Sequence identity indicates a homology with GenBank accesson no. AM233362 (complete genome sequence of *F. tularensis* subsp. *holarctica* LVS).
[2]: Forsman et al. (11).
[3]: Higgins et al. (12).
[4]: Sjöstedt et al. (13).

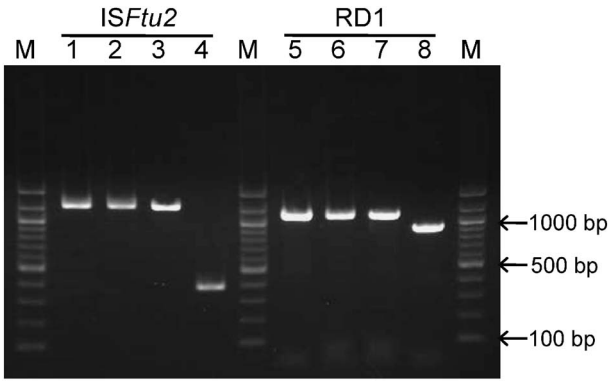

Fig. 1. Identification of *F. tularensis* at the subspecies level. PCR targeting IS*Ftu2* and RD1 was performed using DNA extracted from the pus of the patient (lanes 1 and 5), the trapezius of the hare (lanes 2 and 6), *F. tularensis* subsp. *holarctica* LVS (lanes 3 and 8), *F. tularensis* subsp. *tularensis* Schu strain (lane 4), and the *F. tularensis* subsp. *holarctica* Yama strain, isolated in Japan (lane 7). M, molecular size marker.

Table 2. The number of tandem repeats in 7 loci of *F. tularensis*

Locus	Sample from		Japanese isolate		Reference	
	Patient	Hare	KU-1	NVF1	Fujita et al. (5)	Johansson et al. (6)
Ft-M2	9	9	8	8	2–26	6–26
Ft-M3	3	3	3	3	3–26	3
Ft-M6	4	4	4	4	4, 5	3–5
Ft-M10	5	5	16	5	2–22	4–8
Ft-M18	2	2	2	2	2	2
Ft-M20	8[1]	8[1]	11	11	3–18	11–18
Ft-M25	5	5	5	5	4, 5	5

[1]: Newly found repeat number in this locus.

patient and the dead hare, MLVA genotyping was performed. As shown in Table 2, the *F. tularensis* strains identified in the human and hare samples were indistinguishable; however, they were different from strains KU-1 and NVF1 as well as other previously reported Japanese isolates (5).

The number of human tularemia cases sharply decreased in Japan after the 1960s; thus, tularemia cases are very rare (3). In fact, no tularemia case had been reported for at least approximately 10 years (circa 1999), although five cases of tularemia (including the present case) have emerged since 2008 (17). Four of these five cases were confirmed to have had contact with Japanese hares, thus indicating that *Francisella* spp. are still present in wild animals, hares in particular, in Japan.

In Japan, more than 90% of diagnosed tularemia cases were due to infection by direct contact with either debilitated or dead hares (3), as observed in the present case. However, the contact histories of previous cases were mostly anecdotal, and there has been no detailed study that has addressed the identification of the source of infection. In the present case, the patient was serologically diagnosed with tularemia. Although an attempt to isolate the bacteria failed, both the human patient and hare were found to be infected with *F. tularensis* subsp. *holarctica*, which is endemic to Japan, by PCR (Fig. 1) and direct DNA sequencing performed using the Illumina GAIIx system (Illumina, San Diego, Calif., USA) (9).

We previously reported that MLVA effectively identified 32 genotypes (MLVA pattern) among 33 Japanese *F. tularensis* strains examined (5). In the present study, we determined the MLVA pattern of two additional strains, NVF1 and KU-1, which were independently isolated from dead Japanese hares, in addition to the *F. tularensis*-specific sequences. The genotypes of the newly identified *F. tularensis* strains were different from those reported previously (5,6). These observations confirmed that the *F. tularensis* subsp. *holarctica* in Japan is highly divergent, although the reason for such diversity remains unknown.

MLVA recently conducted in Bulgaria revealed that the buzzard (*Buteo buteo*) also contributes to the transmission of *F. tularensis* to humans, thereby indicating the usefulness of MLVA for identifying the source of infections (18). In this study, we demonstrated that the MLVA patterns from the human and hare samples perfectly matched. Therefore, we concluded that the patient was likely infected from the hare. The MLVA genotyping system can therefore be considered as a powerful tool to better elucidate the epidemiology and ecology of tularemia.

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Conflict of interest None to declare.

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