Surveillance study of bacterial zoonotic diseases among wild animals in Yamaguchi Prefecture, Japan

日本の山口県における野生動物の細菌性人獣共通感染症に関するサーベイランス研究

Joint Graduate School of Veterinary Medicine

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1.PREFACE

Japan, an island nation in East Asia, consists of four major islands—Hokkaido, Honshu, Shikoku, and Kyushu—along with over 3,000 smaller islands, covering an area of 377,727 km². The country stretches more than 2,000 km in length and is largely mountainous, with approximately 75% of its land area covered by mountains. Yamaguchi Prefecture, located at the western tip of Honshu, exemplifies Japan's diverse geography, featuring both the tranquil Seto Inland Sea and a rugged coastline along the Sea of Japan. The region's forests, which cover 70% of the land, provide essential habitats for wildlife that may harbour disease vectors such as ticks and mites. This unique combination of geography and biodiversity presents significant opportunities for studying zoonotic diseases, which are transmitted from animals to humans (35, 68).

Zoonotic diseases are infections transmitted from animals to humans and caused by pathogens like bacteria, viruses, fungi, parasites, and prions. These diseases can spread through contact with infected bodily fluids, animal bites, contaminated water, consuming infected products, and vector bites. Wild animals, livestock, rodents, and birds can serve as hosts for these pathogens (72).

In Japan, approximately 60% of the infectious microorganisms that affect humans, more than 800 species, are transmitted from animals, highlighting the critical need for effective management of zoonoses in public health (22). Japan has largely contained diseases like rabies and plague, and its geographic isolation has played a role in limiting the introduction of new pathogens. However, the increasing importation of animals such as (dogs, cats, foxes, raccoons, and skunks) and animal products, combined with the migration of wild birds, has raised concerns about the potential introduction of new

zoonotic diseases in Japan. Japan's National Epidemiological Surveillance of Infectious Diseases (NESID) monitors these diseases, and recent regulations mandate reporting illnesses such as leptospirosis, tularemia, and avian influenza. To prevent the introduction of new diseases, Japan has enacted strict import controls on certain animals, requiring health certificates for all imported animals. For example, prairie dogs are banned because they can carry zoonotic diseases such as plague and tularemia (22).

The risk of zoonotic disease transmission is heightened by rapid urbanization, land-use changes, and increased interactions between humans and wildlife. In rural areas, growing populations of wild animals such as boars and deer, combined with human encroachment on their habitats, create new pathways for zoonotic diseases to spill over into human populations (25). Therefore, this study focused on the surveillance of zoonotic diseases in wild animals, rodents, and other small mammals, which are thought to play a key role in the maintenance and spread of zoonotic diseases.

Francisella tularensis

Francisella tularensis is the bacterium responsible for tularemia, a zoonotic disease that can infect humans and is classified as a Category A bioterrorism agent due to its high infectivity (33). It was first identified in 1911 in rodents in California and subsequently linked to human cases in Ohio in 1914 (43).

Tularemia is endemic in parts of Europe, northern and central Asia, and North America. There are two primary subspecies that cause human illness: *F. tularensis* subsp. *tularensis*, which is mainly found in North America, and *F. tularensis* subsp. *holarctica*, widespread

across the Northern Hemisphere and Australia. The latter is divided into three biovars: I, II, and japonica (26).

In Europe and Russia, the epidemiology of tularemia is influenced by factors such as geography, wildlife, and human activities (4). In Sweden, mosquitoes are associated with transmission (7), while in Bulgaria and Turkey, food or water contaminated by rodents is a common source of infection. In France and Germany, hunting, especially of hares, leads to ulceroglandular infections (4). In Japan, most cases are mild, and the *F. tularensis* subsp. *holarctica* biovar japonica differs distinctly from other regions in the world (46, 21).

Diagnosis of tularemia involves isolating the bacterium from clinical samples, such as ulcer scrapings, lymph node biopsies, or blood cultures, or via serological tests like microagglutination or ELISA, and highly sensitive genomic DNA detection methods such as PCR (8).

There is no approved vaccine for tularemia, so prevention relies on personal protective measures, including wearing protective clothing and gloves when handling animals, avoiding untreated water, and promptly removing ticks (8). Treatment typically involves intravenous gentamicin for severe cases, with fluoroquinolones such as ciprofloxacin being effective for mild infections (5).

Orientia tsutsugamushi

Orientia tsutsugamushi (OT), the causative agent of scrub typhus, is a Gram-negative, obligate intracellular bacterium that infects various cell types, including macrophages, monocytes, and endothelial cells, and has been detected in cardiac muscle cells and the liver and spleen (37). Scrub typhus was first documented in China in 313 (48) and later described in Japan in 1810 (31).

Scrub typhus is prevalent in the Asia-Pacific region, extending from Korea to Papua New Guinea and parts of India, Afghanistan, and Australia. In addition, patients have also been reported in parts of South Asia, Africa, the Middle East, and South America in recent years (67). It is estimated that around 1 million cases occur annually, affecting over 1 billion people. The disease is typically found in tropical scrublands, primarily affecting rural populations, although travellers in endemic areas are also at risk. The bacterium has several serotypes, with Kato, Karp, and Gilliam being the most globally distributed, along with additional local serotypes in various regions (48).

The disease is transmitted by the bites of larval *Trombiculid* mites (chiggers), which serve as both vectors and reservoirs for OT (67). The incubation period is from 5 to 14 days, and symptoms include fever, headache, rash, and eschar. Severe, untreated cases can result in multi-organ failure and death (27).

Diagnosis of scrub typhus relies on immunofluorescence and immunoperoxidase assays, which are the most reliable methods, as well as ELISA tests. While the Weil-Felix test is still used in endemic regions, it has lower sensitivity and specificity. PCR amplification of the 56-kDa protein gene is also commonly used for detection (67).

Since there is no vaccine for scrub typhus, prevention is an important measure. Recommended preventive measures include applying DEET-based repellents, treating clothing with permethrin, wearing protective clothing (long sleeves, trousers, and closed-toe shoes), and avoiding areas where chiggers are prevalent (1). For treatment, antibiotics such as doxycycline and azithromycin are effective in managing the infection (1).

Tularemia and scrub typhus are classified as Category IV infectious diseases in Japan. Tularemia is endemic to the Tohoku region, and less than ten human cases have been reported in the past decade in Japan (41), while Yamaguchi Prefecture had no reported cases during the past decade. In contrast, approximately 500 human cases of scrub typhus occur annually in Japan (23). Over the last decade, Yamaguchi Prefecture has reported only three cases of scrub typhus, a notably low figure compared to its neighbouring prefectures, and there has been no description of an epidemiological study of the disease in the region previously (24).

This study aimed to explore the ecology of two bacterial zoonotic diseases (*Francisella tularensis* and *Orientia tsutsugamushi*) among wild animals in Yamaguchi Prefecture, focusing on their epidemiology, transmission routes, and impacts on both wildlife and human health.

2.CHAPTER 1 Molecular and serological investigation of *Francisella tularensis* among wild animals in Yamaguchi Prefecture

2.1. Abstract

Francisella tularensis is an intracellular gram-negative bacterium known as the causative agent of tularemia, which can be transmitted to humans by direct contact with wild animals or by tick bites. Although *F. tularensis* is highly pathogenic, its recent prevalence in Japan is underreported due to the small number of reported cases. To clarify the current situation of *F. tularensis* in wild animals, we conducted surveillance on various species of wild animals in Yamaguchi prefecture. In this study, we screened 809 samples collected from 90 Japanese black bears, 105 Japanese monkeys, 168 sika deer, 205 wild boars, and 84 bats. For seroprevalence analysis, we tested 177 serum samples from 75 black bears and 102 monkeys using the microagglutination test. The results showed that serums from five black bears exhibited slight agglutination. Western blot was performed as a confirmatory test on these five samples, but no positive signals were detected. Additionally, molecular surveillance was conducted using DNA extracted from 464 whole blood and 168 tissues, targeting the gene encoding 23 KDa hypothetical protein by real-time PCR and outer membrane protein A gene by conventional PCR. No positive samples of *F. tularensis* were detected by either real-time or conventional PCR. Although we did not detect any *F. tularensis*-positive samples through serological and molecular analyses, continuous surveillance studies are necessary since sporadic human cases have been reported in Japan.

2.2. Introduction

Francisella tularensis is an intracellular Gram-negative bacterium responsible for an infectious zoonotic disease known as tularemia. The bacterium *F. tularensis* has the potential to be used as a weapon of biological warfare agents. Mammals can become infected by direct contact with infected animals, arthropod infestation mainly ticks, drinking contaminated water, eating contaminated food, or inhaling infectious aerosols (8). The clinical presentation of the disease among humans differs according to the mode of transmission and the virulence of the infecting strain (8). Human tularemia is classified into six clinical categories based on the route of infection: 1) ulceroglandular which is transmitted by vectors' bite or direct contact with infected animals, 2) glandular which the infection route is the same as the ulceroglandular except for the absence of ulcer formation, 3) pneumonic which shows most serious illness caused by inhalation of the infected agent, 4) oropharyngeal which is caused by drinking contaminated water or ingestion of contaminated food, 5) oculoglandular which is caused by exposure of the contaminated aerosols to eyes 6) typhoidal which causes general symptoms without localized signs (47, 54).

The *Francisellaceae* family belongs to the γ-subclass of proteobacteria and has only one genus, *Francisella* (34), The causative agent of tularemia has been classified into four subspecies: *F. tularensis* subspecies *tularensis* the most virulent subspecies is mainly reported in North America, *F. tularensis* subspecies *holarctica* is moderately virulent and mainly spread in the northern hemisphere and Eurasia, *F. tularensis* subspecies *novicida* and *mediasiatica* were reported in North America and Australia, and central Asia, respectively. The two subspecies *tularensis* and *holarctica* are pathogenic

to humans (26). In the United States and Europe, the annual incidence rate of human tularemia ranges between 0.5~5/million and 0.3/million, respectively (9, 17).

In Japan, only subspecies of *holarctica* has been reported. From 2003 to 2022, eight human cases of tularemia were officially reported in Japan (41).

Since tularemia is a rare disease and difficult to diagnose, it could be speculated that some patients could be misdiagnosed with other more common illnesses.

In non-primate animals, the severity of this disease differs between animal species. Highly susceptible animals like rodents and lagomorphs are severely affected by infection, resulting in death (19). Although domestic animals, such as cats, dogs, and cows, are comparatively resistant to infection, sheep show high mortality by *F. tularensis* subspecies *tularensis* infection (10, 19, 36). The pathogenicity or clinical investigation of tularemia in wild animals is not well understood. Nevertheless, several sero-epidemiological surveys have been reported. The specific antibodies against *F. tularensis* were detected in wild boars, brown hares, raccoon dogs, and foxes in Germany, and from raccoon dogs, red foxes, and wolves in Sweden, and from foxes, polar bears, and voles in USA (2, 18, 29, 38, 56). In addition, molecular epidemiological research using PCR revealed the detection of partial DNA fragments of *F. tularensis* from Leporidae in Algeria (3).

In Japan, a seroprevalence survey was conducted on wild animal species: Japanese black bears, Japanese hares, Japanese monkeys, rodents, raptors, Japanese raccoon dogs, masked palm civets, and Japanese red foxes, collected between 2002 and 2010. In these animals, specific antibodies for *F. tularensis* were detected from Japanese black bears (15.3%, 23/150), Japanese raccoon dogs (14.2%, 3/21), and field mice (0.8%, 1/120), (52). In addition, molecular surveillance of wild animals, Japanese hares, rats, field mice, voles,

and shrews were conducted in the northern part of Japan. The result showed that partial DNA fragments of *F. tularensis* were detected from Japanese hare only (2%, 1/50), (21). From these previous studies, Japanese hare was thought of as susceptible animals, and Japanese black bears and Japanese raccoon dogs have been identified as potential tularemia sentinel animals in Japan (52).

In our previous serological surveillance, we found a seropositive case of tularemia from a mole collected in Yamaguchi prefecture, in 2015 (data not shown). Since Yamaguchi is a non-endemic area and active surveillance has not been conducted recently, we conducted the surveillance using more than 800 wild animal samples to clarify the presence or absence of tularemia in Yamaguchi prefecture.

2.3. Materials and methods

2.3.1. Samples collection

Samples were collected from wild animals in Yamaguchi prefecture from 2013 to 2022. In this study, we collected samples from a total of 652 individual wild animals. The quadrupedal animals, 90 Japanese black bears (*Ursus thibetanus japonicus*), 105 Japanese monkeys (*Macaca fuscata*), 168 sika deer (*Cervus nippon*), 205 wild boars (*Sus scrofa leucomystax*) were examined. These animals were hunter-harvested or culled for nuisance control under the Program of Prevention from the Birds and Animal Damages in Yamaguchi Prefecture. In addition, 84 bats were collected using a sweep net with an extended rod under the permission of Yamaguchi prefecture, Japan (Prefectural Government Approval R3-429-1 and R4-165-1). In this study, we collected whole blood samples and/or serum samples from quadrupedal animals and liver and spleen samples from bats.

For DNA detection from blood samples, we collected a total of 464 whole blood from 60 black bears, 31 monkeys, 168 sika deer, and 205 wild boars. Also, 177 serum samples were collected from 75 black bears and 102 monkeys in Yamaguchi prefecture (Table 2.1). In bats, tissue samples were collected after euthanasia using isoflurane under the approval of the Animal Research Committee of Yamaguchi University (Permit Number: 550). Both tissue samples were collected from a total of 84 individuals: 36 *Miniopterus schreibersi*, 15 *Myotis macrodactylus*, 6 *Rhinolophus cornutus*, and 27 *Rhinolophus ferrumequinum* (Table 2.1). The distribution of the samples according to the collecting seasons is shown in (Table 2.2).

2.3.2. Sample Processing

Whole blood and serum samples were collected directly from the heart by sterile needles and dispensed in EDTA tubes and tubes free of anticoagulants, respectively. DNAs from 1 mL of whole blood or bat tissues were purified using the Wizard Genomic DNA Purification System Kit (Promega, Madison, WI, USA) or the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's guidance, respectively. Genomic DNA and serum samples were stored at under -30°C.

2.3.3. Molecular detection of *Francisella tularensis*

DNAs from 464 whole blood and 168 tissues were screened by real-time PCR targeting the gene encoding 23 KDa hypothetical protein (Accession No. Y08861), which was reported as an induced protein secreted following macrophage infection with *F. tularensis* (63). Real-time PCR was conducted by the premix Ex Taq PCR kit (probe qPCR) (Takara Bio Inc., Shiga, Japan) with previously described primers and probe (23F, 23R, and 23TM) (55). The reaction was performed in 20 μL volume as duplicate, and the PCR condition was as follows: 95°C for 20 sec followed by 45 cycles of 95°C for 5 sec, 51°C for 15 sec, and 60°C for 20 sec. The positive control used was the genomic DNA of *F. tularensis* subsp. *holarctica* strain LVS and the negative control was a no-template control. Real-time PCR was conducted by the LightCycler 96 PCR system (Roche Diagnostics Corp., Basel, Switzerland). The samples that showed positive results from real-time PCR were confirmed by conventional PCR targeting the *F. tularensis* outer membrane protein A. The conventional PCR was performed by Tks Gflex DNA Polymerase kit (Takara Bio Inc.) following the previous report with minor modification at an annealing temperature of 55°C for 15 sec (58).

2.3.4. Microscopic agglutination (MA) test

Francisella tularensis subspecies *holarctica* (Yama strain) was propagated on chocolate agar (II) (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C for 72 hr. The bacteria were collected using PBS, and after centrifugation, they were inactivated with 5% formalin in 9% saline. The optical density (OD) of bacterial suspension was adjusted to OD_{560}=1.0. The preparation of antigen was examined following the National Institute of Infectious Diseases protocol 2nd edition with minor modifications (42). A volume of 25 μL of 2-fold serial dilutions of an inactivated serum was mixed with an equal volume of formalin-inactivated bacteria stained with 0.005% safranin (49). The reaction was performed in a 96-well round-bottom microtiter plate (Watson, Tokyo, Japan) and was observed after overnight incubation at 37°C. In this study, we used anti-*F. tularensis* macaque serum as the positive control and the normal human serum (Tennessee Blood Services, Memphis, TN, USA) as a negative control (62). The maximum serum dilution displaying agglutination with the antigen was used to express the titer. Bacterial agglutination at serial dilutions of 1:10 or higher was considered MA positive (20, 51, 52).

2.3.5. Lipopolysaccharide purification

Lipopolysaccharide (LPS) was purified from *F. tularensis* subspecies *holarctica* (NVF1 strain), a Japanese isolate (13). The bacterium was cultured at 37°C for 72 hr on chocolate agar (II) plates and then collected using PBS, and the suspension was adjusted to an OD_{600}=1.2. After centrifugation, it was re-suspended in the lysis buffer of the LPS extraction kit (iNtRON Biotechnology, Kyungki-Do, Korea). The bacterial cells were

inactivated at 65°C for 10 min. LPS was extracted using the previously mentioned kit following the manufacturer's protocol.

2.3.6. SDS-PAGE and Western blotting

Purified LPS was separated in 5-20% gradient gel (SuperSep Ace, Fujifilm Wako pure chemicals corporation, Osaka, Japan) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) by immunoblotting. The membrane was then blocked in 3% skim milk with PBS containing 0.1% Tween 20 at room temperature for 2 hr (52). Inactivated serum samples were diluted to a ratio of 1:1000 and incubated with the membrane at room temperature overnight (20). The Anti-*F. tularensis* LPS monoclonal antibody clone FB 11 (HyTest, Turku, Finland) was used as positive control (1:1000). The membrane was washed three times with PBS containing 0.1% Tween 20 and then incubated with the second antibody at room temperature 2 hr. The second antibody of serum or positive control were HRP-conjugated recombinant A/G protein (Thermo Fisher Scientific, Waltham, MA, USA., 1:8000) or HRP conjugated goat anti-mouse IgG & IgM antibody (Thermo Fisher Scientific, 1:8000), respectively. The reaction was detected using Clarity western ECL substrate (Bio-Rad Laboratories) and visualized by the Amersham ImageQuant 800 (Cytiva, Tokyo, Japan).

2.4. Results

2.4.1. DNA detection from blood and tissues

In this study, we tested a total of 632 DNA samples for the detection of *F. tularensis* DNA using real-time PCR. Thirty-five samples were inconclusive result, with a high Cq value (>35) by real-time PCR. These samples were confirmed by conventional PCR. As a result, none of the samples were positive (Table 2.3).

2.4.2. Seroprevalence

Serum samples from 177 wild animals were tested for the presence of *F. tularensis* antibodies using the MA test. The results revealed that five samples from black bears showed slight or partial agglutination of the *F. tularensis* subspecies *holarctica* (Yama strain) antigen at serially diluted serum (1:10), while no agglutination was observed in the monkey samples. Confirmatory tests were conducted using the western blot analysis for the samples that showed positive signals by the MA test. However, the results of the western blot showed no ladder-like band detected in all five samples (Supplementary Figure 2.1).

2.5. Discussion

Tularemia is a zoonotic bacterial disease that greatly impacts animal and human health. Several research studies have been conducted to investigate the source of infection in nature. In the world, it has been reported that about 30 species of birds, more than 100 species of mammals, and 100 species of arthropods were associated with tularemia. Because of this diversity in nature, it is unlikely that the disease will disappear from the natural environment, and it is speculated that cases are latent even in areas where no patients have been observed. In Japan, animal cases have been reported since the 1930s, mainly in Fukushima prefecture and other areas in the Tohoku region. Nevertheless, there have been few epidemiological studies in the western part of Japan, especially in wild animals (12). In this study, we conducted sero-epidemiological and molecular-epidemiological surveillance using more than 800 samples collected from several wild animals in Yamaguchi prefecture. This is the first tularemia surveillance study conducted on wild animals in Yamaguchi prefecture, which is a non-endemic area for tularemia. In this study, we did not detect any positive cases. Considering that 12.9% seroprevalence has been reported from wild bears in Iwate prefecture, which has a high number of reported patients, the result of this study is consistent with the actual situation that no patients have been reported so far in Yamaguchi prefecture (20).

Since Yamaguchi prefecture is a non-endemic area for tularemia, we needed to conduct a study using highly susceptible animals and large number of samples to assess the potential of tularemia. In a previous study conducted in Japan, bear showed high seroprevalence (20). We collected 90 bear samples and tested seroprevalence and the antigen by PCR. Although the results were negative, we could not collect foxes or other small predators, which are indicator animals for tularemia in the world (18, 29, 52, 56).

Further analysis is needed to understand their role in tularemia epidemiology in non-endemic regions like Yamaguchi prefecture.

The MA test is the golden standard test for the detection of antibodies against tularemia in the serum. Our results of MA test revealed that five bear samples showed slightly positive results, but considering the limitations of the MA test in cross-reactivity with other bacteria, western blotting was conducted, and the results were negative (Supplementary Figure 2.1 and Table 2.3). False positive results of the MA test could occur due to infection of a bacterium closely related to *F. tularensis*. Hotta et al. described a false positive reaction of the MA test using wild bear serum (20). Similarly, our results indicated occasional positive results in bear samples, suggesting the possibility that the bears were infected with a bacterium similar to *F. tularensis*.

Although all of the samples tested negative in this study, continued surveillance in susceptible animals, such as foxes, Japanese raccoon dogs or wild bears, will be important to monitor human cases in the future. Additionally, we attempted to detect *F. tularensis* antigens from several wild animal species, including four bat species, although the results were negative. Nevertheless, this kind of comprehensive survey involving various wild animal species should be continued from the perspective of early detection of unknown pathogens.

Overall, this is the first comprehensive surveillance study to understand the current situation of *F. tularensis* among wild animals in Yamaguchi prefecture. Although the serological and molecular analyses did not reveal any *F. tularensis*-positive samples, our findings across various wild animal species still serve as important baseline information, given that reservoir species of *F. tularensis* are diverse. In addition, this study focused only on *F. tularensis*, and we could not exclude the presence of other

Francisella spp. and *Francisella*-like species. Future studies should consider this aspect and it is important to investigate other wild animal species that could be the indicator animals.

2.6. Figures and tables

Table 2.1 The total number of individual animal species and sample types used in this study.

Animal species	Number of individuals	Number of DNA samples [*1]	Number of serum samples
Japanese black bear (*Ursus thibetanus japonicus*)	**90**	**60**	**75**
Japanese monkey (*Macaca fuscata*)	**105**	**31**	**102**
sika deer (*Cervus nippon*)	**168**	**168**	**0**
wild boar (*Sus scrofa leucomystax*)	**205**	**205**	**0**
Bats	**84**	**168**	**0**
Total	**652**	**632**	**177**

[*1] DNA purified from whole blood of quadrupedal animals, or spleen and liver of bats

Table 2.2 The collecting season of the wild animal samples used in this study.

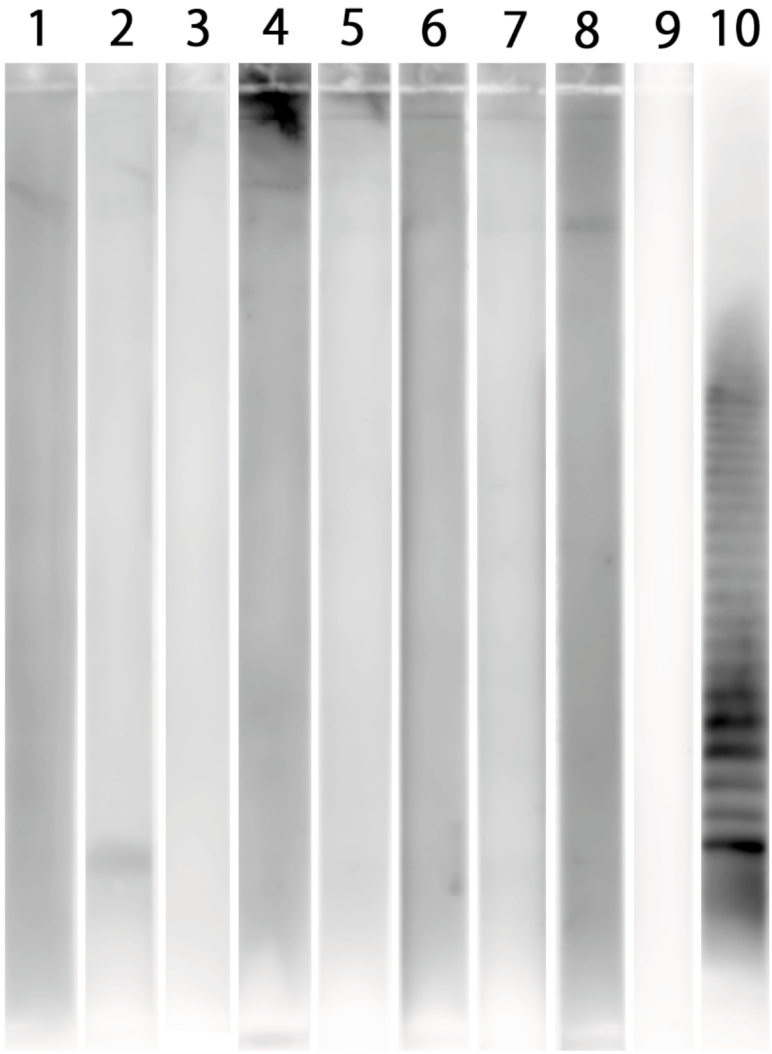
Animal Species	Number of animals	Winter (December, January, and February)	Spring (March, April, and May)	Summer (June, July, and August)	Autumn (September, October, and November)
Japanese black bear	90	3 (3.3%)	13 (14.4%)	23 (25.6%)	51 (56.7%)
Japanese monkey	105	30 (28.6%)	6 (5.7%)	45 (42.8%)	24 (22.8%)
sika deer	168	62 (36.9%)	30 (17.9%)	28 (16.6%)	48 (28.5%)
wild boar	205	84 (40.9%)	40 (19.5%)	33 (16.1%)	48 (23.4%)
Bat	84	50 (59.5%)	0 (0%)	10 (11.9%)	24 (28.6%)

Table 2.3 The result of DNA and antigen detection

Animal species	Number of individuals	Results/samples		
		PCR	MA test	Western blot
Japanese black bear	**90**	**0/60**	**5/75**	**0/5**
Japanese monkey	**105**	**0/31**	**0/102**	**NT**
sika deer	**168**	**0/168**	**NT**	**NT**
wild boar	**205**	**0/205**	**NT**	**NT**
Bat	**84**	**0/168**	**NT**	**NT**
Total	**652**	**0/632**	**5/177**	**0/5**

NT: not tested.

Supplementary Figure 2.1



Supplementary Figure 2.1 Detection of antibodies to *F. tularensis* by western blot.

The MA test positive 5 samples were examined by western blot analysis (lanes 1-5). The MA test negative 3 bear serum (lane 6-8), serum blank control (lane 9), and anti-*F. tularensis* LPS monoclonal antibody FB11 (positive control; lane 10) were shown.

3. CHAPTER 2 The high prevalence of *Orientia tsutsugamushi* among wild rodents and the identification of its major serotypes in Yamaguchi prefecture, Japan, where scrub typhus patients are rarely identified

3.1. Abstract

Orientia tsutsugamushi (OT) is an obligate intracellular bacterium transmitted by larval trombiculid mites, responsible for scrub typhus in humans. In Japan, approximately 500 human cases are reported annually, with six major serotypes identified as Kato, Karp, Gilliam, Irie/Kawasaki, Shimokoshi, and Hirano/Kuroki. However, the prevalence of OT in Yamaguchi prefecture remains largely unknown. This study aimed to investigate the prevalence and serotypes of OT in wild rodents collected from eight locations within the prefecture. DNA was extracted from spleen and liver samples of 135 wild rodents collected between 2015 and 2024, and PCR was conducted to detect OT targeting the 56 kDa type-specific antigen gene. As a result, five individuals (3.7 %) were detected positive for OT DNA. OT was successfully isolated from two of the five rodents and characterized by multi-locus sequence analysis (MLSA) based on 11 housekeeping genes. The MLSA results indicated that both isolates clustered with OT strain Ikeda (Japanese Gilliam serotype). Additionally, we performed a serological test on 117 serum samples from wild rodents using the indirect immune peroxidase test. The results showed that 59.8 % (n=70/117) of the rodents had antibodies against OT, with 73% (n=51/70) showing the highest titer against the OT strains Gilliam (Gilliam serotype) and Ikeda (Japanese Gilliam serotype), known to be transmitted by *Leptotrombidum pallidum* mites. Overall, the present study identified the OT serotypes and potential primary vector species in Yamaguchi prefecture, emphasizing the need for further surveillance, particularly in humans.

3.2. Introduction

Orientia tsutsugamushi (OT) is a gram-negative, obligate intracellular bacterium belonging to the family *Rickettsiaceae*. This family includes other notable genera, such as *Rickettsia*, which are also intracellular bacteria. OT causes scrub typhus, which is also known as Tsutsugamushi disease, a mite-borne zoonotic disease in humans (27, 64, 67, 48). Humans are infected with OT through the bites of larvae of Chigger mites (family *Trombiculidae*), which serve as both vectors and reservoirs for OT (27, 64). The incubation period for the disease is 5 –14 days (67, 6). Typical clinical symptoms of scrub typhus were fever, headache, rash, and eschar. Severe cases can lead to multi-organ failure and death if untreated with suitable antibiotics. The disease is endemic within the tsutsugamushi triangle, encompassing northern Japan and far-eastern Russia in the north, northern Australia in the south, and Pakistan in the west; however, cases have also been reported outside this region (27, 67). Over a million human cases are documented annually in this region (6).

Chigger mites from the genus *Leptotrombidium* are the primary vectors transmitting OT, while small mammals, particularly rodents, serve as important blood-feeding hosts and sentinel animals of scrub typhus (64, 59, 53). Transmission of OT occurs transstadially and transovarially in mites (67). In Japan, several vector species are associated with the transmission of different OT serotypes: *Leptotrombidium akamushi* (serotype Kato), *L. pallidum* (serotypes Karp and Gilliam), and *L. scutellare* (serotypes Irie/Kawasaki and Hirano/Kuroki) (28). Additionally, it has recently been suggested that *L. pallidum* and *L. palpale* are the candidate vectors of the serotypes Japanese Gilliam (JG) and Shimokoshi, respectively (50, 45).

The geographical distribution of patients and seasonal patterns are correlated with the distribution of vectors (28). For instance, the distribution of *L. akamushi* is limited to the northern part of Japan's main island and is responsible for transmitting OT serotype Kato. Serotypes Irie/Kawasaki and Hirano/Kuroki are transmitted by *L. scutellare*, which is less tolerant of cold weather, and cases are mainly reported in the southern Tohoku and Kyushu regions. In contrast, *L. pallidum* can tolerate cold weather and transmit serotypes Karp, JG, and Gilliam throughout Japan. In addition, sporadic cases of OT serotype Shimokoshi have been reported in several prefectures, with *L. palpale* being the candidate vector (50).

In Japan, over 6,000 cases of scrub typhus were reported between 2007 and 2021 (40), with the highest number recently recorded in Kagoshima, Fukushima, Miyazaki, and Chiba prefectures (24). In these endemic regions, two distinct seasonal patterns of patient incidence have been identified: the "spring-summer type", primarily connected to the vector *L. pallidum*, and the "late autumn type", associated with the vector *L. scutellare* (28, 40). The activity and behavior of these vectors are closely correlated with the seasonal peaks of scrub typhus each year. Therefore, identifying OT serotypes and their vectors is crucial for predicting the seasonal occurrence of scrub typhus in specific regions. Over the past decade, only three human cases of scrub typhus have been reported in Yamaguchi prefecture (24). However, this figure may not accurately reflect the true incidence, as the clinical symptoms of scrub typhus often resemble those of other diseases, such as Japanese spotted fever caused by *Rickettsia japonica*, leptospirosis caused by *Leptospira* spp., Q fever caused by *Coxiella burnetii*, and so on. Additionally, around 20 scrub typhus cases were reported yearly in the neighbouring prefectures, Hiroshima and Shimane, suggesting that a similar number of patients may also exist in Yamaguchi

prefecture (32). Hence, this study aimed to investigate the prevalence and endemic serotypes of OT in the region by focusing on small mammals, primarily wild rodents, collected from various locations.

3.3. Materials and methods

3.3.1. Ethical statements

The small wild rodents in this study were collected with permission from the Yamaguchi prefecture in Japan (Prefectural Government Approval Nos. 435-1, R2-129, R4-277, and R6-53). The tissue and serum sampling from wild rodents and the *in vivo* isolation of OT were approved by the Animal Care and Use Committee of Yamaguchi University (Approval Nos. 494, 581, and 600). The *in vivo* and *in vitro* isolation of OT were conducted in the Biosafety Level 3 Facility at the Joint Faculty of Veterinary Medicine, Yamaguchi University.

3.3.2. Sample collection and DNA extraction

Wild rodents were captured using Sherman traps from eight areas (Yamaguchi University Yoshida campus, Nakao, Niho, Suzenji, Kano, Atou, Hirakawa, and Akiyoshi) in Yamaguchi prefecture between 2015 and 2024 (Figure 3.1). Traps were set in the forestry and rural areas, avoiding the private lands such as residential and farming areas. Captured rodents were transported to the university for species identification and sample collection. Euthanasia was performed with isoflurane inhalation followed promptly by cardiac puncture blood sampling. Body weight and morphological measurements were taken for species morphological identification before dissection to collect liver and spleen samples. Tissue samples were stored at -80 °C until used for bacterial isolation. DNA was extracted from liver and spleen samples using the DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol for DNA isolation. Extracted DNA and serum samples were kept at -30 °C or -80 °C until further use.

3.3.3. Rodent species identification

Molecular identification was conducted following morphological species identification. Two pairs of primers targeting the mitochondrial cytochrome b gene for the identification of *Apodemus* and *Rattus* species, respectively, were designed to amplify a 1,140 bp fragment in PCR (see supplementary Table 1 for the primer sequences used). The PCR reaction was performed in a 25 µl reaction mixture using Tks Gflex DNA Polymerase (Takara Bio, Shiga, Japan), 12.5 µl of 2×Gflex PCR Buffer (Mg^{2+}, dNTP plus), 1 µl of each primer, 8 µl of deionized water, and 2 µl of DNA template. The PCR amplification conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 45 sec, annealing at 54 °C for 30 sec, and extension at 72 °C for 45 sec with a final extension step at 72 °C for 7 min. PCR products were confirmed on 1% agarose gel and purified with the Axygen™ AxyPrep MAG PCR Clean-Up Kit (Axygen Bioscience, Union City, CA, USA) before being sequenced by Eurofins Genomic company. The obtained sequences were compared with public databases using the Nucleotide Basic Local Alignment Search Tool

(https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.4. *Orientia tsutsugamushi* detection

DNA extracted from liver and spleen samples of wild rodents was used for OT screening by nested PCR targeting the 56 kDa type-specific antigen gene following the protocol of Furuya et al. (15). The nested PCR was performed using puReTaq™ Ready-To-Go™ PCR Beads (Cytiva, Tokyo, Japan). The amplified PCR products were confirmed for the size of 483 bp and purified and sequenced as described for rodent species identification. All primers used in this analysis are listed in Supplementary Table 3.1.

3.3.5. *Orientia tsutsugamushi* isolation using experimental mice

Three PCR-positive samples (Rodent IDs: YU19, YU55, and YU130) were selected for OT isolation. The isolation was performed as previously described (70). Briefly, liver or spleen tissues were homogenized with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS (-); Fujifilm, Osaka, Japan), using a BioMasher II (Nippi, Incorporated Protein Engineering, Tokyo, Japan) and inoculated intraperitoneally into six-week-old BALB/c mice (n = 3). The second *in vivo* passage in ICR mice was performed using the same procedure. All experimental mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Inoculated mice were monitored daily for 10 – 12 days for clinical symptoms such as body weight loss, ruffled fur, and lethargy. At the end of the experiment, mice were humanely euthanized in accordance with the approval of the Animal Care and Use Committee of Yamaguchi University for sample collection.

3.3.6. *In vitro* isolation

The liver and spleen samples collected from the *in vivo* experiment were individually homogenized as described above using Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, MA, USA). Homogenates were then centrifuged, and the supernatant was inoculated into a culture of murine fibroblast (L929) cells, which were propagated in DMEM medium (supplemented with 10% fetal bovine serum). In addition, ascitic fluid was diluted with DMEM before being applied to L929 cells. Cell cultures were monitored daily for cytopathic effects, and OT particles were observed using a dark field microscope. Infected cells were passaged onto fresh cultured cells, and the supernatant was collected for DNA extraction using DNeasy Blood and Tissue Kits (Qiagen). To confirm the isolates, PCR targeting the 56 kDa antigen gene was performed

using the TaKaRa *Ex Taq* (Takara Bio), which amplified a 1,003 bp internal fragment of the gene, followed by sequencing of the products.

3.3.7. Multi-locus sequencing analysis (MLSA)

Two isolates from rodent IDs YU19 and YU130 were subjected to MLSA by targeting 11 housekeeping genes (Supplementary Table 3.1) previously published by Nakayama et al. (39). Amplifications were performed using TaKaRa *Ex Taq* or KOD FX Neo (Toyobo, Osaka, Japan). All amplified products were confirmed for their respective sizes before purification and sequencing, as described above.

3.3.8. Phylogenetic analysis

The sequences of the 56 kDa antigen gene and the 11 MLSA genes obtained in this study were used in separate for phylogenetic analyses using the neighbor-joining method under the Tamura-Nei model in MEGA version X (30). All sequences are available in the GenBank database under accession numbers LC846340 to LC846366. The details of accession numbers for MLSA are provided in Supplementary Table 3.3.

3.3.9. Serological testing

To detect the presence of anti-OT antibodies in the serum of wild rodents, an indirect immune-peroxidase test (IPT) was conducted. Antigen-coated slides containing antigen spots of OT strains Karp, Kato, Gilliam, Kawasaki, Kuroki, Shimokoshi, Ikeda (JG), and 402I (Japanese Karp subtype 2) were prepared (45). The IPT was performed following the protocol previously described (69, 57). The serum samples were serially diluted fourfold (1:40, 1:160, 1:640, 1:2560, 1:10240, 1:40960) starting from an initial dilution

of 1:10 in diluent containing 0.3% bovine serum albumin in 0.01M phosphate-buffered saline (0.01M PBS, pH 7.2). A total of 20 ul of the diluted serum was applied to each antigen-coated well, and the slides were incubated at 37 °C for 30 min in a humidity box. Following the incubation, the slides were washed twice with the washing buffer (0.01M PBS). The slides were then treated with a secondary antibody, peroxidase-conjugated AffiniPure Goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch Lab., Inc., PA, USA), diluted 1:100, and incubated at 37 °C for 30 min in the humidity box, followed by twice washing. A chromogenic substrate solution was prepared by combining 4-chloro-1-naphthol (Fujifilm) in 80 % ethanol with 3 % hydrogen peroxide (Kenei Pharmaceutical Co., Ltd., Osaka, Japan). Finally, the slides were incubated in this solution at 37 °C for 10 min. Subsequently, the slides were washed three times with distilled water and dried before being covered with coverslips using a mounting medium (Kaizers glycerol gelatin, Merck, Darmstadt, Germany). The slides were then examined under a light microscope with a 100x magnification. The assay result was graded as positive if a positive signal was detected, with the titer recorded as the highest dilution factor of 1:40 or greater (Supplementary Table 3.2).

3.4. Results

3.4.1. Sample collection

A total of 135 wild small mammals were captured from eight areas during the sampling period, predominantly consisting of rodent species identified morphologically and molecularly as *Apodemus speciosus, A. argenteus*, *Rattus rattus*, *Mus musculus*, and *Eothenomys smithii*. Additionally, one *Crocidura dsinezumi*, a member of the insectivore group, was identified. Details of the rodent species and the collected DNA and serum samples are presented in Table 3.1.

3.4.2. Detection and isolation of *Orientia tsutsugamushi*

A total of 123 and 134 DNA samples extracted from spleen and liver tissues, respectively, were used for OT detection by nested PCR targeting the 56 kDa antigen gene. The results revealed that 3.7% (n = 5/135) of wild rodents (Rodent IDs: YU10, YU19, YU55, YU130, and KM8) were positive for OT. The PCR-positive samples were *A. speciosus* captured in the Yamaguchi University Yoshida campus, Nakao, Suzenji, and Hirakawa areas (Figure 3.1 and Table 3.2). Of the five OT DNA-positive samples, three samples (YU19 from Nakao, YU55 from Hirakawa, and YU130 from Suzenji) were used for OT isolation. As a result, two isolates, YU19 and YU130, were successfully obtained and confirmed by sequencing the 56 kDa antigen gene.

The 483 bp sequences from the rodent samples YU10 and KM8 and the 1,003 bp sequences from the isolates YU19 and YU130 of the internal fragment of the 56 kDa antigen gene showed over 95 % identity to the OT strain Ikeda (JG serotype). Meanwhile, the sequence of the DNA fragment from rodent YU55 showed 99.75 % identity to the serotype Irie/Kawasaki sequence (M63383). Phylogenetic analysis of the 56 kDa antigen

gene revealed that four of the five samples clustered with the strain Ikeda, while YU55 clustered with the strain Kawasaki (Figure 3.2). In addition, 11 housekeeping genes were successfully amplified for MLSA, and phylogenetic analysis of the concatenated sequences confirmed that both isolates formed a phylogenetically identical cluster with the strain Ikeda (Figure 3.3).

3.4.3. Seroprevalence of *Orientia tsutsugamushi* in wild rodents

The results of the IPT revealed a 59.8 % overall positive rate for OT antibodies in wild rodents. Among the different collection areas, rodents collected from Yamaguchi University Yoshida campus showed the highest seroprevalence at 90 %, followed by Nakao (60 %), Niho (59 %), Atou (50 %), Suzenji (46 %), Kano (27 %), and Hirakawa (25 %). No antibodies against OT were detected in rodents from Akiyoshi (Table 3.2). Furthermore, positive samples from Yamaguchi University Yoshida campus showed the highest antibody titers against either the serotype Gilliam or JG, or both (n = 26/27), while one sample (YU 134) showed the same level of antibody titers against serotypes Karp, Kato, and Shimokoshi (Supplementary Table 3.2). Similarly, in Nakao, all positive samples (n = 21/21) exhibited high antibody titers against the serotype Gilliam or JG, or both, with six samples also displaying the same level of antibody titers against serotype Shimokoshi. In Niho, nine positive samples (n = 9/10) demonstrated highest antibody titers against either the serotype Gilliam or JG, or both, while one sample (YU 24) had high antibody titers against Irie/Kawasaki serotype. In Suzenji, four positive samples (n = 4/6) showed the highest antibody titers against either the serotype Gilliam or JG, or both, while two other positive samples (YU 65 and YU 67) had the highest antibody titers against the serotype Shimokoshi. In Kano, one positive sample showed the highest

antibody titers against the serotypes Gilliam and JG, and another showed the highest antibody titers against serotype Shimokoshi, and a third sample exhibited the same level of antibody titers against serotypes Gilliam, JG, and Shimokoshi. All positive samples from Atou (n = 2) and Hirakawa (n = 1) showed the highest antibody titers against both the serotypes Gilliam and JG.

3.5. Discussion

In Japan, scrub typhus was first reported by Hashimoto in Niigata prefecture in 1810 (31). The causative agent *Rickettsia tsutsugamushi* was reclassified in 1995 to a new genus, *Orientia*, and renamed *Orientia tsutsugamushi* (48). The World Health Organization designated it as one of the most underdiagnosed and underreported infections, requiring hospitalization (65). In Japan, scrub typhus was classified as a "Category IV infectious disease" in 1999, necessitating the immediate reporting of confirmed cases (71).

Since different OT serotypes may present in different districts even within the same prefecture (24, 16), it is essential to identify the endemic serotypes and their associated vectors in order to make an accurate diagnosis. In this study, we collected 135 wild rodents from eight locations in Yamaguchi prefecture and detected high seroprevalence against the serotypes Gilliam and JG (Supplementary Table 3.2). Two OT strains were successfully isolated and characterized as the JG serotype based on MLSA. In addition, a small number of wild rodents with the highest antibody titers against the serotype Shimokoshi were captured at several survey sites, suggesting that this serotype may be sporadically present in Yamaguchi prefecture, similar to the neighboring prefecture, Shimane (24). Furthermore, the results of antibody titers and DNA detection indicated a low prevalence of the serotype Irie/Kawasaki. Despite a few reported cases of scrub typhus in Yamaguchi prefecture, no surveillance, including in wild rodents, has been conducted. Thus, these data provide the first insight into the potential OT serotypes circulating in wild rodents in Yamaguchi prefecture.

Human cases have been constantly reported in neighboring prefectures such as Hiroshima and Shimane. For instance, between 2013-2021, 210 human cases were reported in Hiroshima, and 30 cases were reported in Shimane. However, only three human cases

were reported in Yamaguchi prefecture (24). In Hiroshima prefecture, the most commonly reported OT serotypes in patients were Irie/Kawasaki and Karp (24). Specifically, serotype Irie/Kawasaki was found in 94 % (n = 16/17) of patients, while serotype Karp was detected in 6 % (n = 1/17) of patients in Hiroshima City, located in the western part of Hiroshima prefecture (14). In wild rodents, serotypes Karp and Irie/Kawasaki were detected throughout Hiroshima prefecture with a seroprevalence of 34.1 % (n = 56/139) (53). In their study, DNA fragments of OT were detected in 14.6 % (n = 24/164) of wild rodents, with the majority being serotype Karp (n = 23/24).

In contrast, in Shimane prefecture, located to the north of Hiroshima prefecture, most human cases were associated with serotypes Karp and Gilliam, both of which are known to be transmitted by *L. pallidum*. Additionally, DNA fragments of the serotype Karp (n = 5/151) and serotype Gilliam (n = 2/151) were detected in wild rodents (60). Sporadic cases of serotype Shimokoshi, which is transmitted by *L. palpale*, have also been documented in Shimane prefecture (11).

In summary, the serotype Irie/Kawasaki, transmitted by *L. scutellare*, is more prevalent in Hiroshima prefecture, with serotype Karp, transmitted by *L. pallidum*, also reported. Human cases exhibited a bimodal peak, with more cases reported in autumn, likely due to *L. scutellare* being the main vector. In Shimane prefecture, serotype Karp has accounted for the majority of cases, and serotype Gilliam, which is also transmitted by *L. pallidum*, has also been reported. Since *L. pallidum* is the main vector, the number of human cases tends to be higher in spring than in autumn (24).

In this study, IPT and DNA detection revealed that 59.8 % and 3.7 % of wild rodents had antibodies or DNA fragments of OT, respectively. These results were similar to the antibody prevalence in wild rodents from Hiroshima prefecture and the DNA-positive

rate in wild rodents from Shimane prefecture, suggesting a high probability of OT occurrence in Yamaguchi prefecture. Additionally, the major serotypes detected in Yamaguchi prefecture were Gilliam and JG (Figure 3.2 and Supplementary Table 3.2), with two isolated strains characterized as the JG serotype. Since cross-reactivity between Gilliam and JG has previously been observed using the immunofluorescence assay (45), although the vector species was not investigated in this study, our findings suggest that *L. pallidum* may be the predominant vector. Therefore, scrub typhus occurrence in Yamaguchi prefecture is likely to follow a bimodal pattern, with a higher peak in spring, similar to Shimane prefecture. This characteristic should be considered in future surveillance studies in the prefecture.

Research conducted in Japan examining the virulence of OT serotypes and subtypes revealed that Kato, JG, Gilliam, and Karp exhibited high virulence in mice. In contrast, the Japanese Karp 2 subtype showed intermediate virulence, and the Japanese Karp 1 subtype, Kawasaki and Kuroki exhibited low virulence (45). Furthermore, more severe cases have been reported in humans infected with serotypes transmitted by *L. pallidum* (61). Therefore, the high seropositivity rate of OT and the identification of *L. pallidum*-transmitted serotypes in serum samples from wild rodents suggest the presence of the OT transmission cycle in Yamaguchi prefecture, and that the number of human cases reported in the prefecture over the past decade likely does not reflect the current situation of scrub typhus.

Another notable finding from the IPT is that the central area of Yamaguchi prefecture, including Yamaguchi University Yoshida campus, Niho, and Nakao, had a relatively higher prevalence of antibodies against OT compared to other areas, such as Atou, Suzenji, Kano, and Akiyoshi (Table 3.2). In particular, Yamaguchi University Yoshida campus is

characterized by fragmented forests, which may have resulted in a lower number of animal hosts compared to other area (Figure 3.1, Table 3.2). Increased biodiversity can reduce disease transmission in multi-host or multi-vector systems, as different hosts and vectors exhibit varying degrees of transmission competence (44), a phenomenon known as the dilution effect. This concept has been discussed in research examining the ecology of Lyme disease and the roles of its vectors (66). This may help explain the high prevalence (90 %) of OT among wild rodents at Yamaguchi University Yoshida campus. However, the sample size and sampling period might have influenced the results of this study, and more comprehensive investigations are needed to confirm these findings.

Our study demonstrated a high seroprevalence of the serotypes Gilliam and JG among wild rodents, with the successful isolation of OT serotype JG. These findings imply the presence of a transmission cycle for serotypes Gilliam and JG in Yamaguchi prefecture. Furthermore, *L. pallidum* could be the primary vector species responsible for OT transmission in the prefecture. Future studies incorporating surveillance of human serum samples will be essential for validating these findings.

3.6. Figures legends

Figure 3.1 Yamaguchi prefecture map showing the eight sampling locations.

Figure 3.2 Phylogenetic tree based on 56 kDa type-specific gene of *Orientia tsutsugamushi*.

The phylogenetic tree was constructed in MEGA version X by the Neighbor-joining model with the Tamura-Nei parameter and 250 bootstrap replications. The sequences obtained in this study are indicated in red.

Figure 3.3 Phylogenetic tree based on MLSA of *Orientia tsutsugamushi*.

The phylogenetic tree was generated using the concatenated sequences of 11 housekeeping genes in the order of *atbD, clpX*, *dnaJ*, *dnaK*, *fabD*, *gyrB, icd, mdh, nrdA, sucD*, and *ubiD*, according to the Nakayama et al. (2010). The Neighbor-joining model was used with the Tamura-Nei parameter and 250 bootstrap replications for phylogenetic construction in MEGA version X. The sequences obtained in this study, YU19 and YU130 isolates, are shown in red.

3.7. Figures and tables

Figure 3.1

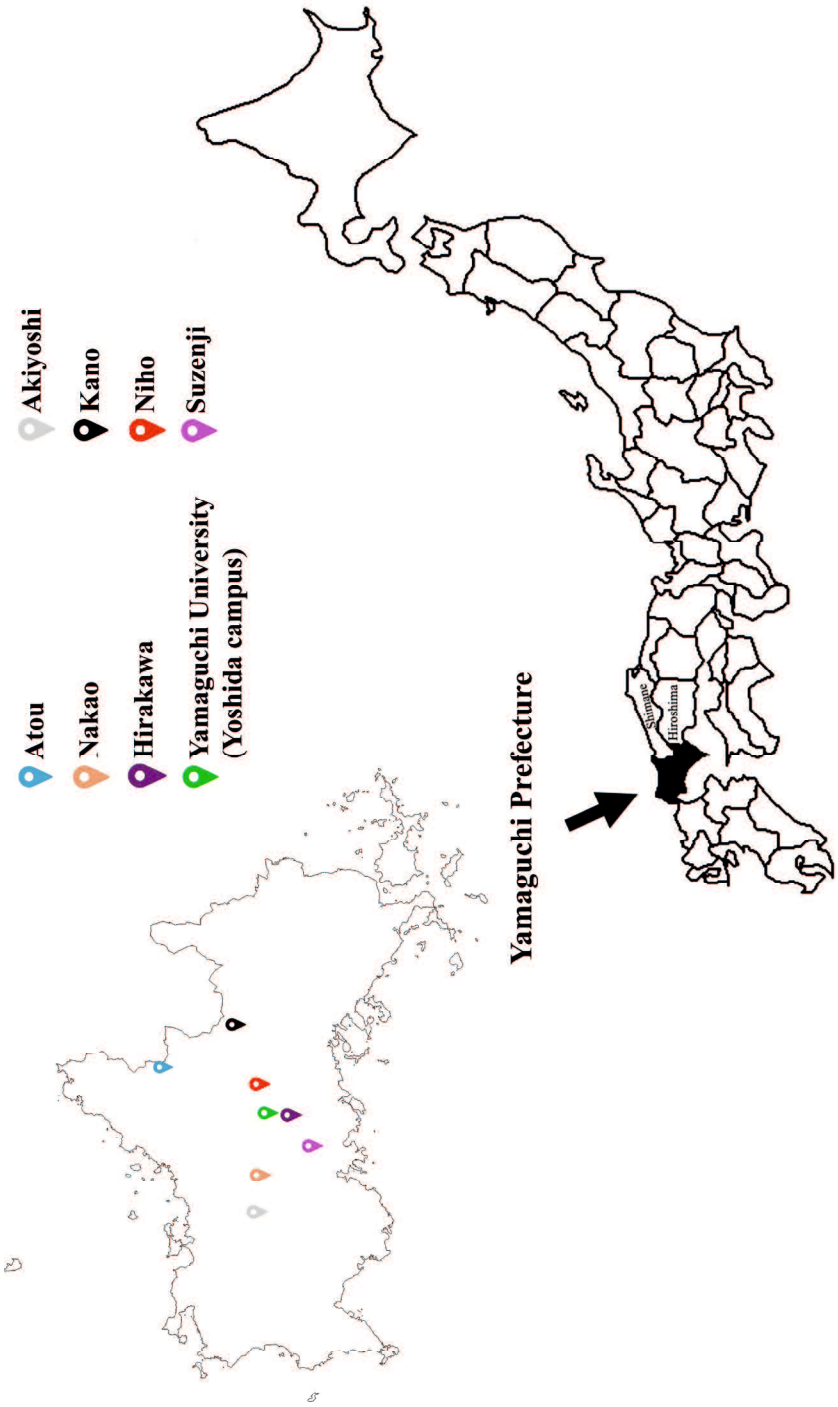


Figure 3.2

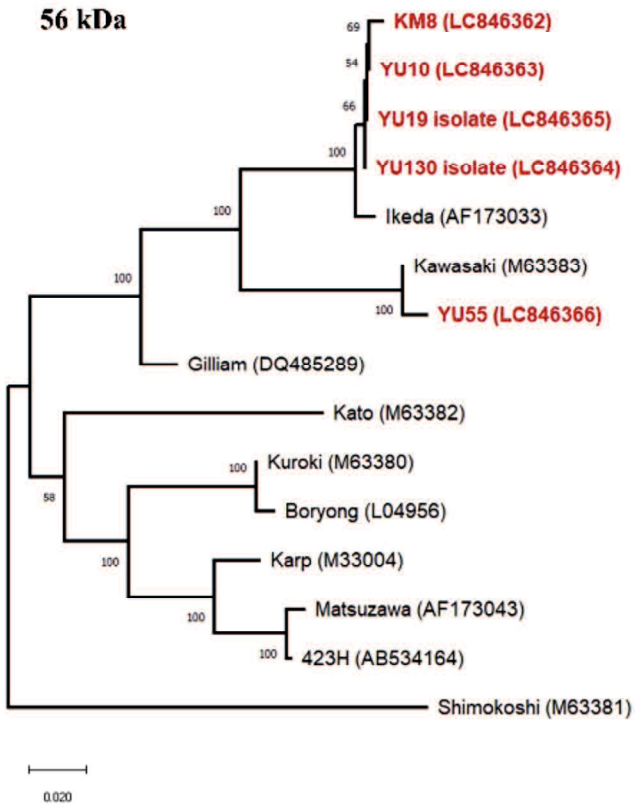

56 kDa
KM8 (LC846362)
YU10 (LC846363)
YU19 isolate (LC846365)
YU130 isolate (LC846364)
Ikeda (AF173033)
Kawasaki (M63383)
YU55 (LC846366)
Gilliam (DQ485289)
Kato (M63382)
Kuroki (M63380)
Boryong (L04956)
Karp (M33004)
Matsuzawa (AF173043)
423H (AB534164)
Shimokoshi (M63381)
69
54
66
100
100
100
100
100
58
100
100
100
0.020

Figure 3.3

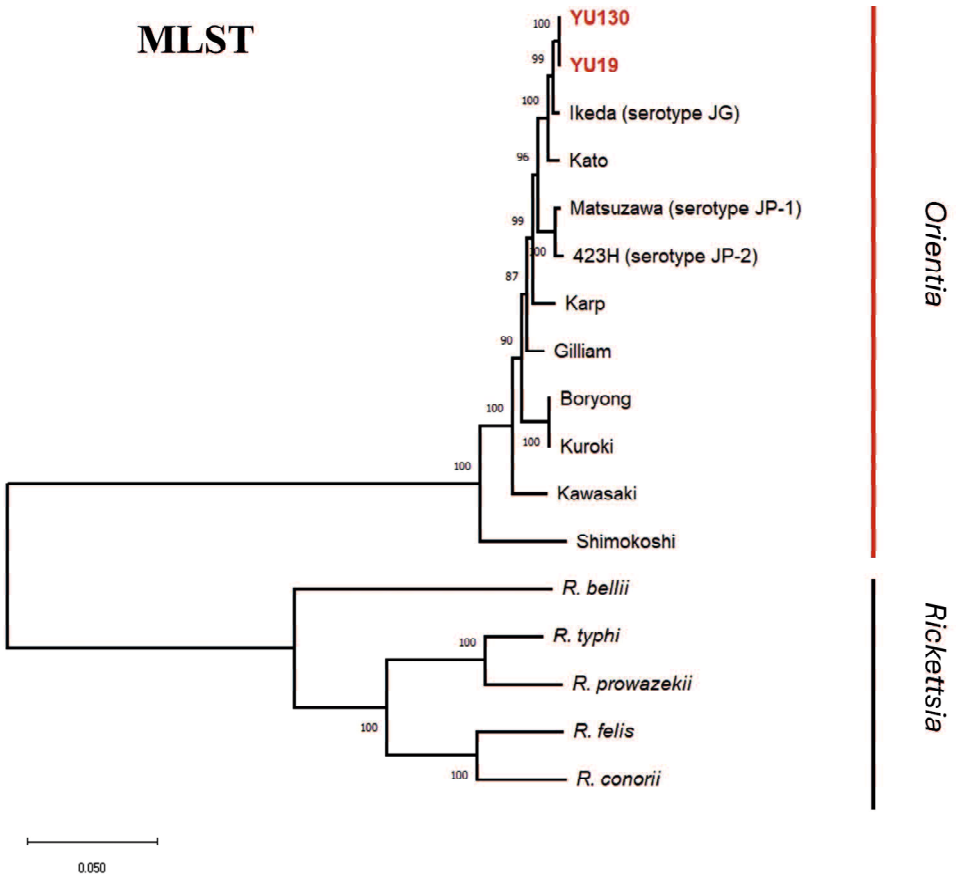

MLST
YU130
YU19
Ikeda (serotype JG)
Kato
Matsuzawa (serotype JP-1)
423H (serotype JP-2)
Karp
Gilliam
Boryong
Kuroki
Kawasaki
Shimokoshi
R. bellii
R. typhi
R. prowazekii
R. felis
R. conorii
Orientia
Rickettsia
100
99
100
96
99
100
87
90
100
100
100
100
100
100
0.050

Table 3.1 The details of rodent species and collected samples in this study.

Animal species	Number of individuals	Spleen (DNA)	Liver (DNA)	Serum
Apodemus speciosus	102	99	101	93
Apodemus argenteus	25	16	25	22
Rattus rattus	4	4	4	0
Mus musculus	1	1	1	0
Crocidura dsinezumi	1	1	1	0
Eothenomys smithii	2	2	2	2
Total	**135**	**123**	**134**	**117**

Table 3.2 The prevalence of the *Orientia tsutsugamushi* by collection site.

Collection site	DNA detection (56 kDa antigen gene)	Serological detection (Indirect immunoperoxidase test)
Yamaguchi University (Yoshida campus)	3 % (1/39)	90 % (27/30)
Nakao	5 % (2/38)	60 % (21/35)
Niho	0 % (0/18)	59 % (10/17)
Suzenji	7 % (1/15)	46 % (6/13)
Kano	0 % (0/14)	27 % (3/11)
Atou	0 % (0/4)	50 % (2/4)
Hirakawa	25 % (1/4)	25 % (1/4)
Akiyoshi	0 % (0/3)	0 % (0/3)
Total	**3.7 % (5/135)**	**59.8 % (70/117)**

Supplementary table 3.1 Primers used in this study.

Primer Name	Sequence	Target Gene	Annealing tempreture	Amplified fragment	Reference
Detection of OT					
1st outer primers		56 kDa type-specific antigen gene	57 °C	1003 bp	Furuya et al.,1993
34'	TCAAGCTTATTGCTAGTGCAATGTCTGC				
55'	AGGGATCCCTGCTGCTGTGCTTGCTGCG				
2nd inner primers			57 °C	483 bp	
10'	GATCAAGCTTCCTCAGCCTACTATAATGCC				
11'	CTAGGGATCCCGACAGATGCACTATTAGGC				
MLSA of OT isolates					
atpD_F	TCTGCAGTTGGTTATCAGCC	*atpD* (ATP synthase beta-chain)	50 °C	459 bp	Nakayama et al., 2010
atpD_R	ATACTACCAACCATGTAAAAAGC				
clpX_F	TTAATGTACCATTTGCAATGGC	*clpX* (ATP-dependent Clp protease ATP-binding subunit)	50 °C	435 bp	
clpX_R	TAGCAATTACAGGTAATCTACC				
dnaJ_F	TCTCAAGTATCATCCTGATC	*dnaJ* (DnaJ protein)	50 °C	546 bp	
dnaJ_R	GTTTTACCTCCTCTACTTCC				
dnaK_F	AGTAGCGCTTGGAGCTGC	*dnaK* (DnaK protein)	55 °C	522 bp	
dnaK_R	GCTTTAATAGCTTGCTGCAG				
fabD_F	AGATTATGTAGCTGGCCATTC	*fabD* (malonyl CoA-acyl carrier protein transacylase)	50 °C	480 bp	
fabD_R	AACTTTGCCAGGACCTATTTC				
gyrB_F	ATGTTAAATTTTTTTATACTGGTGG	*gyrB* (DNA gyrase subunit B)	50 °C, 55 °C	477 bp	
gyrB_R	GCTCGTTGTTGACAATCTGC				
icd_F	AAGACTATGCTATGTTTGAAGC	*icd* (isocitrate dehydrogenase)	50 °C, 55 °C	489 bp	
icd_R	ATAAAACGACAACACCAATGATC				
mdh_F	ATAAAGCAAAAATTTCCATAATAGG	*mdh* (malate dehydrogenase)	50 °C, 55 °C	348 bp	
mdh_R	TCAAGTACTCCAGCCATTCC				
nrdA_F	CTGAGCATCATCAAAAATTAGG	*nrdA* (ribonucleoside-diphosphate reductase alpha-chain)	50 °C	603 bp	
nrdA_R	TAAATGTTGCACTGAGCCTTC				
sucD_F	TCTATATTTGAAGCTATTGATGC	*sucD* (succinyl-CoA synthetase alpha-chain)	50 °C, 55 °C	432 bp	
sucD_R	ATTATTGCTCCAGCATGTCCC				
ubiD_F	ATCAGAATGAAGGACCTTTTGG	*ubiD* (3-octaprenyl-4-hydroxybenzoate carboxy-lyase)	50 °C	456 bp	
ubiD_R	CCCATTTCCTTGTAGTTTCAGG				
***Apodemus* species identification**					
Forward	AGCYATACACTAYACATCAGA	Mitochondrial cytochrome b gene	54 °C	1140 bp	This study
Reverse	GTKGCTTTRTCTACTGAGAA				
***Rattus* species identification**					
Forward	CCCATCYAACATCTCATCA	Mitochondrial cytochrome b gene	54 °C	1140 bp	This study
Revirse	TTTGATCCTGTTTCGTGGAGGA				

Supplementary table 3.2 The result of IPT titres.

				Indirect immunoperoxidase test							
Location	Rodent ID	Rodent species	Date of collection	Gilliam	JG (Ikeda)	Karp	JP-2 (402I)	Kato	Irie/ Kawasaki	Hirano/ Kuroki	Shimokoshi
Yamaguchi University (Yoshida campus)	YU 1	*Apodemus speciosus*	2016.05.01	5120	640	1280	2560	2560	1280	1280	1280
	YU 2	*Apodemus speciosus*	2016.05.03	5120	10240	2560	1280	1280	5120	1280	2560
	YU 3	*Apodemus speciosus*	2016.05.08	80	40	40	-	40	40	-	-
	YU 4	*Apodemus speciosus*	2016.06.14	1280	1280	320	80	640	160	40	160
	YU 5	*Apodemus speciosus*	2016.06.14	1280	1280	320	160	320	320	-	1280
	YU 6	*Apodemus speciosus*	2016.07.06	320	640	80	40	40	160	-	160
	YU 7	*Apodemus speciosus*	2016.07.15	5120	5120	80	80	1280	5120	40	320
	YU 8	*Apodemus speciosus*	2016.07.15	2560	2560	160	640	160	640	640	160
	YU 9	*Apodemus speciosus*	2016.07.15	2560	1280	640	80	1280	640	80	80
	YU 10	*Apodemus speciosus*	2016.07.20	640	640	320	160	160	640	40	160
	YU 11	*Apodemus speciosus*	2016.07.20	640	640	40	40	40	80	-	40
	YU 12	*Apodemus speciosus*	2017.02.07	40	160	-	-	-	80	-	-
	YU 13	*Apodemus speciosus*	2017.02.28	160	160	-	40	40	-	40	40
	YU 14	*Apodemus speciosus*	2017.02.28	1280	1280	640	80	640	320	640	640
	YU 15	*Apodemus speciosus*	2017.03.23	1280	1280	1280	640	320	640	320	320
	YU 56	*Apodemus speciosus*	2018.08.15	320	320	80	80	80	80	-	80
	YU 57	*Apodemus speciosus*	2018.08.17	1280	1280	40	40	80	80	-	640
	YU 58	*Apodemus speciosus*	2018.08.30	1280	5120	80	50	80	80	-	1280
	YU 59	*Apodemus speciosus*	2018.09.22	1280	1280	80	80	80	80	-	80
	YU 62	*Apodemus speciosus*	2018.11.09	5120	10240	2560	1280	2560	1280	40	5120
	YU 63	*Apodemus speciosus*	2018.11.29	5120	5120	320	320	80	1280	40	1280
	YU 84	*Apodemus speciosus*	2022.10.12	2560	2560	320	160	320	1280	-	320
	YU 85	*Apodemus speciosus*	2022.10.12	-	-	-	-	-	-	-	-
	YU 107	*Apodemus speciosus*	2023.06.28	320	640	160	320	160	320	-	160
	YU 108	*Apodemus speciosus*	2023.06.28	-	-	-	-	-	-	-	-
	YU 109	*Apodemus speciosus*	2023.06.28	-	160	-	-	-	-	-	-
	YU 110	*Apodemus speciosus*	2023.06.28	160	160	-	80	-	40	-	80
	YU 133	*Apodemus speciosus*	2024.06.26	-	-	-	-	-	-	-	-
	YU 134	*Apodemus speciosus*	2024.06.26	-	-	40	-	40	-	-	40
	YU 135	*Apodemus speciosus*	2024.06.26	1280	640	80	640	160	640	160	160

Nakao	KM 2	*Apodemus argenteus*	2015.04.05	1280	1280	160	320	80	160	-	640
	KM 3	*Apodemus argenteus*	2015.04.05	320	320	80	40	80	40	-	160
	KM 4	*Apodemus argenteus*	2015.04.05	640	640	80	40	320	40	-	320
	KM 5	*Apodemus argenteus*	2015.04.05	320	320	160	80	160	160	-	320
	KM 6	*Apodemus argenteus*	2015.04.06	320	320	80	40	80	40	-	80
	KM 7	*Apodemus argenteus*	2015.04.06	320	320	40	40	-	40	-	80
	KM 8	*Apodemus speciosus*	2015.04.06	1280	2560	320	320	320	160	40	1280
	KM 9	*Apodemus argenteus*	2015.04.06	80	80	-	-	-	-	-	80
	KM 10	*Apodemus speciosus*	2015.04.06	640	640	320	320	320	320	40	320
	KM 11	*Apodemus argenteus*	2015.04.06	-	-	-	-	-	-	-	-
	KM 12	*Eothenomys smithii*	2015.04.06	-	-	-	-	-	-	-	-
	KM 13	*Apodemus speciosus*	2015.04.06	320	320	80	40	80	80	-	80
	YU 16	*Apodemus speciosus*	2017.05.17	-	-	-	-	-	-	-	-
	YU 17	*Apodemus speciosus*	2017.05.17	-	-	-	-	-	-	-	-
	YU 18	*Apodemus speciosus*	2017.05.17	160	160	40	40	160	40	-	160
	YU 19	*Apodemus speciosus*	2017.05.17	640	640	320	320	640	320	-	640
	YU 20	*Apodemus speciosus*	2017.05.17	-	-	-	-	-	-	-	-
	YU 46	*Apodemus speciosus*	2018.04.17	320	320	320	160	-	-	-	320
	YU 47	*Apodemus speciosus*	2018.04.17	80	80	40	-	40	-	-	40
	YU 48	*Apodemus speciosus*	2018.04.17	-	-	-	-	-	-	-	-
	YU 49	*Apodemus speciosus*	2018.04.17	-	-	-	-	-	-	-	-
	YU 50	*Apodemus speciosus*	2018.04.17	-	-	-	-	-	-	-	-
	YU 51	*Apodemus speciosus*	2018.04.17	-	-	-	-	-	-	-	-
	YU 52	*Apodemus speciosus*	2018.04.17	2560	2560	-	-	320	320	-	80
	YU 53	*Apodemus speciosus*	2018.04.17	80	80	-	-	40	-	-	40
	YU 61	*Apodemus speciosus*	2018.11.07	2560	2560	320	320	80	320	-	320
	YU 70	*Apodemus speciosus*	2019.03.28	2560	640	40	160	640	160	40	160
	YU 71	*Apodemus speciosus*	2019.03.28	320	640	40	40	80	160	-	80
	YU 72	*Apodemus speciosus*	2019.03.28	320	-	40	-	160	-	-	40
	YU 73	*Apodemus speciosus*	2019.03.28	1280	40	80	40	640	160	-	1280
	YU 74	*Apodemus speciosus*	2019.03.28	-	-	-	-	-	-	-	-
	YU 117	*Apodemus speciosus*	2024.05.08	-	-	-	-	-	-	-	-
	YU 118	*Apodemus argenteus*	2024.05.08	-	-	-	-	-	-	-	-
	YU 119	*Apodemus speciosus*	2024.05.10	-	-	-	-	-	-	-	-
	YU 120	*Apodemus speciosus*	2024.05.10	-	-	-	-	-	-	-	-

Suzenji	YU 64	*Apodemus speciosus*	2018.12.19	-	-	-	-	-	-	-	-
	YU 65	*Apodemus speciosus*	2019.03.27	40	40	80	-	80	-	-	160
	YU 66	*Apodemus speciosus*	2019.03.27	320	640	320	320	80	320	-	320
	YU 67	*Apodemus speciosus*	2019.03.27	320	80	80	40	40	40	-	640
	YU 69	*Apodemus speciosus*	2019.03.27	-	-	-	-	-	-	-	-
	YU 125	*Apodemus speciosus*	2024.05.23	40	40	-	-	-	40	-	-
	YU 126	*Apodemus speciosus*	2024.05.23	-	-	-	-	-	-	-	-
	YU 127	*Apodemus speciosus*	2024.05.23	-	-	-	-	-	-	-	-
	YU 128	*Apodemus speciosus*	2024.05.24	-	-	-	-	-	-	-	-
	YU 129	*Apodemus speciosus*	2024.05.24	40	40	-	-	-	40	-	-
	YU 130	*Apodemus speciosus*	2024.05.24	-	-	-	-	-	-	-	-
	YU 131	*Apodemus speciosus*	2024.05.24	1280	1280	320	80	320	80	-	320
	YU 132	*Apodemus speciosus*	2024.05.24	-	-	-	-	-	-	-	-

Niho	YU 21	*Apodemus argenteus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 22	*Apodemus speciosus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 23	*Apodemus argenteus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 24	*Apodemus speciosus*	2017.06.14	1280	2560	1280	320	2560	5120	1280	1280
	YU 25	*Apodemus argenteus*	2017.06.14	1280	1280	160	80	80	80	-	160
	YU 26	*Apodemus speciosus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 27	*Apodemus speciosus*	2017.06.14	5120	5120	320	320	1280	160	-	640
	YU 28	*Apodemus speciosus*	2017.06.14	1280	320	80	160	80	80	-	320
	YU 29	*Apodemus speciosus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 30	*Apodemus speciosus*	2017.06.14	10240	5120	640	640	1280	2560	-	1280
	YU 31	*Apodemus speciosus*	2017.06.14	1280	2560	1280	320	1280	640	-	2560
	YU 32	*Apodemus argenteus*	2017.06.14	160	160	-	-	-	-	-	-
	YU 33	*Apodemus argenteus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 34	*Apodemus speciosus*	2017.06.14	640	640	320	160	320	160	-	320
	YU 36	*Apodemus speciosus*	2017.06.14	2560	2560	80	320	320	160	40	320
	YU 37	*Apodemus speciosus*	2017.06.14	-	-	-	-	-	-	-	-
	YU 38	*Apodemus speciosus*	2017.06.14	80	160	40	40	40	80	-	40

Kano	KM 14	*Eothenomys smithii*	2015.04.28	-	-	-	-	-	-	-	-
	KM 15	*Apodemus argenteus*	2015.04.28	1280	1280	-	-	-	-	-	320
	KM 16	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 17	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 18	*Apodemus speciosus*	2015.04.28	80	80	-	40	80	40	-	160
	KM 20	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 21	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 23	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 24	*Apodemus speciosus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 25	*Apodemus argenteus*	2015.04.28	-	-	-	-	-	-	-	-
	KM 26	*Apodemus speciosus*	2015.04.28	160	160	40	40	-	40	-	160
Atou	YU 39	*Apodemus argenteus*	2017.07.14	-	-	-	-	-	-	-	-
	YU 40	*Apodemus speciosus*	2017.07.14	2560	2560	160	80	640	40	-	1280
	YU 41	*Apodemus speciosus*	2017.07.14	160	320	40	-	40	40	-	80
	YU 42	*Apodemus speciosus*	2017.07.14	-	-	-	-	-	-	-	-
Hirakawa	YU 55	*Apodemus speciosus*	2018.05.01	1280	1280	320	80	320	640	-	320
	YU 121	*Apodemus speciosus*	2024.05.15	-	-	-	-	-	-	-	-
	YU 122	*Apodemus speciosus*	2024.05.16	-	-	-	-	-	-	-	-
	YU 123	*Apodemus speciosus*	2024.05.16	-	-	-	-	-	-	-	-
Akiyoshi	YU 43	*Apodemus speciosus*	2017.07.14	-	-	-	-	-	-	-	-
	YU 44	*Apodemus speciosus*	2017.07.14	-	-	-	-	-	-	-	-
	YU 45	*Apodemus speciosus*	2017.08.20	-	-	-	-	-	-	-	-

Supplementary table 3.3 Accession numbers used for MLSA phylogenetic tree

Species and strains	*atbD*	*clpX*	*dnaJ*	*dnaK*	*fabD*	*gyrB*	*icd*	*mdh*	*nrdA*	*sucD*	*ubiD*
***Orientia tsutsugamushi* YU 19**	LC846340	LC846341	LC846342	LC846343	LC846344	LC846345	LC846346	LC846347	LC846348	LC846349	LC846350
***Orientia tsutsugamushi* YU 130**	LC846351	LC846352	LC846353	LC846354	LC846355	LC846356	LC846357	LC846358	LC846359	LC846360	LC846361
***Orientia tsutsugamushi* Gilliam**	AB537251	AB537259	AB537315	AB537323	AB537243	AB537267	AB537275	AB537283	AB537291	AB537299	AB537307
***Orientia tsutsugamushi* Karp**	AB537248	AB537256	AB537312	AB537320	AB537240	AB537264	AB537272	AB537280	AB537288	AB537296	AB537304
***Orientia tsutsugamushi* Kato**	AB537253	AB537261	AB537317	AB537325	AB537245	AB537269	AB537277	AB537285	AB537293	AB537301	AB537309
***Orientia tsutsugamushi* Kawasaki**	AB537249	AB537257	AB537313	AB537321	AB537241	AB537265	AB537273	AB537281	AB537289	AB537297	AB537305
***Orientia tsutsugamushi* Kuroki**	AB537250	AB537258	AB537314	AB537322	AB537242	AB537266	AB537274	AB537282	AB537290	AB537298	AB537306
***Orientia tsutsugamushi* Shimokoshi**	AB537252	AB537260	AB537316	AB537324	AB537244	AB537268	AB537276	AB537284	AB537292	AB537300	AB537308
***Orientia tsutsugamushi* Matsuzawa**	AB537255	AB537263	AB537319	AB537327	AB537247	AB537271	AB537279	AB537287	AB537295	AB537303	AB537311
***Orientia tsutsugamushi* 423H**	AB537254	AB537262	AB537318	AB537326	AB537246	AB537270	AB537278	AB537286	AB537294	AB537302	AB537310
***Orientia tsutsugamushi* Ikeda**	AP008981										
***Orientia tsutsugamushi* Boryong**	AM494475										
Rickettsia bellii	CP000087										
Rickettsia typhi	AE017197										
Rickettsia felis	CP000053										
Rickettsia conorii	AE006914										
Rickettsia prowazekii	AJ235273	AJ235272	AJ235270	AJ235270	AJ235273	AJ235272	AJ235271	AJ235271	AJ235272	AJ235271	AJ235273

4. GENERAL CONCLUSION

Zoonoses with wildlife reservoirs involve various transmission routes, highlighting the need for improved national surveillance systems for both humans and animals, as well as enhanced international collaboration and information sharing. Continuous evaluation of reportable diseases is crucial for effectively addressing public health concerns. Zoonotic agents can be transmitted directly from animals to humans, via vectors such as ticks and mosquitoes, or through the consumption of animal products, emphasizing the need for robust monitoring and response strategies.

In Chapter 1, we conducted molecular and serological surveillance of *Francisella tularensis* among various wild animal species in Yamaguchi Prefecture. We analyzed 809 samples from 90 Japanese black bears, 105 Japanese monkeys, 168 sika deer, 205 wild boars, and 84 bats, as well as 177 serum samples from 75 black bears and 102 monkeys. Although no *F. tularensis*-positive samples were detected, these findings provide valuable baseline information considering the diverse reservoir species for the bacterium. The study focused exclusively on *F. tularensis*, leaving the presence of other *Francisella* species or similar organisms unaddressed, which future research should explore, particularly in other wild animal species that may serve as indicators.

In Chapter 2, we investigated the prevalence and serotypes of *Orientia tsutsugamushi* in six species of wild rodents from eight locations across Yamaguchi Prefecture. Molecular analysis on 135 rodents collected between 2015 and 2024 revealed that five rodents (3.7%) tested positive for *O. tsutsugamushi* DNA, Two *O. tsutsugamushi* strains, YU19 and YU130, were successfully isolated and characterized by multi-locus sequence analysis

(MLSA) based on 11 housekeeping genes, and were classified as the Ikeda strain (Japanese Gilliam serotype). Additionally, serological testing of 117 serum samples from wild rodents using the indirect immune peroxidase test showed the highest titers against the Gilliam and Japanese Gilliam serotypes, which are known to be transmitted by *Leptotrombidum pallidum* mites. This study focused on identifying *O. tsutsugamushi* serotypes and potential primary vector species in Yamaguchi Prefecture.

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