



Seroepidemiological Survey of Zoonotic Diseases in Small Mammals with PCR Detection of *Orientia tsutsugamushi* in Chiggers, Gwangju, Korea

Jung Wook Park¹, Jae Keun Chung¹, Sun Hee Kim¹, Sun Ju Cho¹, Yi Deun Ha¹, So Hyang Jung¹, Hye Jung Park¹, Hyun Jae Song², Jung Yoon Lee², Dong Min Kim³, Jah Pyus³, Dong Ryong Ha¹, Eun Sun Kim¹, Jae Il Lee^{4,*}

¹Division of Infectious Disease Investigation, Health and Environment Research Institute of Gwangju City, Gwangju 61986, Korea; ²Clinical Pathology, Gwangju Health University, Gwangju 62287, Korea; ³Department of Internal Medicine, School of Medicine, Chosun University, Gwangju 61452, Korea; ⁴Department of Veterinary Public Health, College of Veterinary Medicine, Chonnam National University, Gwangju 62287, Korea

Abstract: Serosurveillance for zoonotic diseases in small mammals and detection of chiggers, the vector of *Orientia tsutsugamushi*, were conducted from September 2014 to August 2015 in Gwangju Metropolitan Area. *Apodemus agrarius* was the most commonly collected small mammals (158; 91.8%), followed by *Myodes regulus* (8; 4.6%), and *Crocidura lasiura* (6; 3.5%). The highest seroprevalence of small mammals for *O. tsutsugamushi* (41; 26.3%) was followed by hantaviruses (24; 15.4%), *Rickettsia* spp. (22; 14.1%), and *Leptospira* (2; 1.3%). A total of 3,194 chiggers were collected from small mammals, and 1,236 of 3,194 chiggers were identified with 7 species of 3 genera: *Leptotrombidium scutellare* was the most commonly collected species (585; 47.3%), followed by *L. orientale* (422; 34.1%), *Euchoengastia koreaensis* (99; 8.0%), *L. palpale* (58; 4.7%), *L. pallidum* (36; 2.9%), *Neotrombicula gardellai* (28; 2.3%), and *L. zetum* (8; 0.6%). *L. scutellare* was the predominant species. Three of 1,236 chigger mites were positive for *O. tsutsugamushi* by PCR. As a result of phylogenetic analysis, the *O. tsutsugamushi* strain of chigger mites had sequence homology of 90.1-98.2% with Boryong. This study provides baseline data on the distribution of zoonotic diseases and potential vectors for the development of prevention strategies of vector borne diseases in Gwangju metropolitan area.

Key words: *Leptotrombidium scutellare*, *Leptotrombidium orientale*, *Orientia tsutsugamushi*, zoonotic disease, serosurveillance, rodent, soricomorph, chigger

INTRODUCTION

Zoonotic diseases in small mammals are a risk to humans who work or conduct recreational activities in field environments. During the fall period, typical zoonotic diseases in the Republic of Korea (ROK) are scrub typhus, hantaviruses, and leptospirosis [1]. They annually occur and cause acute, febrile illness to humans. Scrub typhus especially became increasingly important. According to the Korea Centers for Disease Control and Prevention (KCDC), chigger mite distribution has expanded from the south to central Korea. The number of scrub typhus patients increased from approximately 200 cases in the early 1990s, to >10,000 cases in 2013 [1]. Known species of chigger mites include *Leptotrombidium pallidum*, *L. scutellare*, *L.*

palpale, *L. orientale*, *L. zetum*, *Neotrombicula gardellai*, *Euchoengastia koreaensis*, *Neotrombicula japonica* in the ROK. *L. pallidum* is the predominant species in the central region, and *L. scutellare* in the southern region [2]. In the world, there have been more than 20 antigenically distinct strains of scrub typhus reported [3]. The prototype Kato, Karp, and Gilliam strains were classified into a higher virulence group for humans. The Boryong type, which is a major strain in ROK, was classified into a lower one [4].

Recent studies emphasized the relationships between a warming climate and distribution of primary scrub typhus vectors [5,6], as well as detection of *O. tsutsugamushi* positive chigger mites, including new species as potential vectors of *O. tsutsugamushi* [7]. Therefore, this investigation focused on scrub typhus and other zoonotic diseases among small mammals and associated *O. tsutsugamushi* positive chigger mites. This information will provide baseline data for future investigation and information for development of zoonotic diseases mitigation strategies.

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*Corresponding author (jaeil@chonnam.ac.kr)

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MATERIALS AND METHODS

Collection site

From September 2014 to August 2015, small mammals were collected using Sherman live traps (3"×3.5"×9", USA) in 2 regions (Gwangsan-gu and Buk-gu) of Gwangju Metropolitan Area (MA), ROK. Collection sites were just within the city perimeter. The 2 regions where traps were installed were in a similar environment. Both included 5 types of locations. The locations were fallow ground, a ridge between rice fields, a boundary between forest and field, around tombs, and around water. Traps were baited with biscuits covered with peanut butter and set up in the late afternoon, and collected from 8-10 a.m, the following morning. Gwangju MA surrounded by Chonnam Province is a densely populated area (Fig. 1).

Collection of rodents and chiggers

Traps positive for small mammals were numbered, placed into secure shipping containers, and then transported to the Health and Environment Research Institute of Gwangju MA.

Small mammals were euthanized using chloroform (Merck, West Point, Pennsylvania, USA) soaked cotton (1×1 cm). Then, blood was centrifuged at 3,000 rpm for 20 min, and serum was separated and maintained at 4°C. Indirect immunofluorescence assay test (IFAT) and passive hemagglutination assay (PHA) were performed 24-36 hr after the small mammals were euthanized for antibody detection. The carcasses of the small mammals were hung over a glass bowl containing water to harvest chiggers, and chiggers were collected the following day.

Detection of *O. tsutsugamushi*, hantantaviruses, and *Rickettia* spp. antibodies in small mammals

A total of 10 µl of sera from each small mammal was used for serial dilutions of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1,024, and 1:2,048 in PBS (pH 7.2). Diluted sera were deposited on an antigen spot slide, incubated at 37°C for 30 min in a humidified chamber, and then washed as in step 2. First, the slide was washed for 3 min with PBS, and second, washed for 3 min with distilled water to remove PBS salt. A total of 25

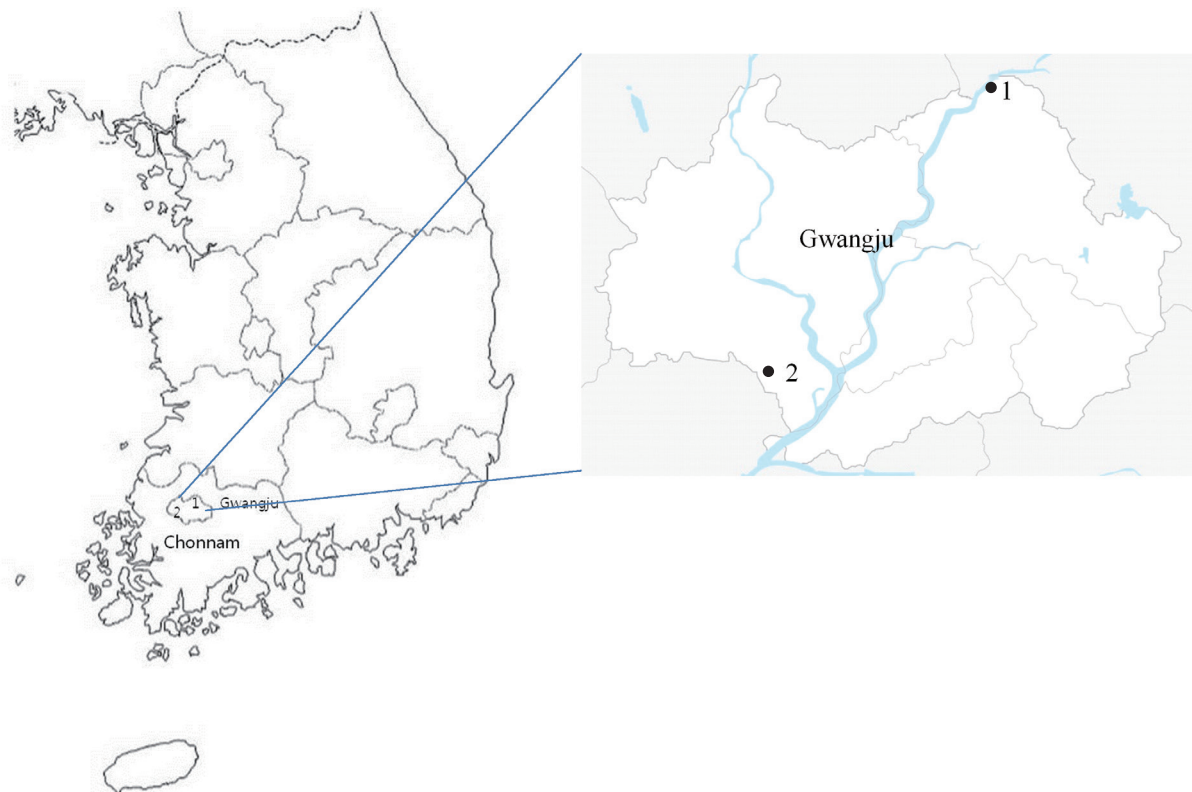


Fig. 1. Map of wild rodent collection sites. 1. Buk-gu (N 35° 13' 51.7", E 126° 54' 23.8"), 2. Gwangsan-gu (N 35° 09' 19.2", E 126° 45' 05.4") in Gwangju Metropolitan Area (MA), Republic of Korea.

µl fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, St. Louis, Missouri, USA) was added to a spot slide, and the slides were incubated at 37°C for 30 min in a humidified chamber. The slides were washed for 3 min with PBS, distilled water, and then air-dried. After mounting medium (Sigma) was added to a spot slide, and covered with coverslip, the slides were examined for specific spots using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). A cut-off titer of 1:16 was used to identify seropositivity. Antigen spot slides for *O. tsutsugamushi*, hantaviruses, and *Rickettsia* spp. were provided by KCDC.

Detection of *Leptospira* spp. antibodies in small mammals

Genedia Lepto PHA (Green Cross, Seoul, Korea) kit reagents were used for detection of leptospirosis by PHA. A total of 25 µl serum from each small mammal was added to a 96-well plate, and diluted 1:80 in a dissolved solution of Genedia Lep-to kit. A total of 75 µl of sheep blood containing red blood cells sensitized by *Leptospira* spp. was placed on a diluted serum for agglutination assay. Ag-Ab agglutination reaction in 1:80 tested positive for leptospirosis.

Detection of *O. tsutsugamushi* in chigger mites by PCR

The method used by Ree et al. [8] was applied for the detection of *O. tsutsugamushi* from individual chiggers. Individual chiggers were placed on a glass slide with PBS 10 µl. The chigger's internal contents were squeezed out with 2 fine pins and observed after suspension with 50 µl PBS under stereomicroscope (Carl Zeiss). The chigger exoskeleton was mounted with polyvinylalcohol medium (Bioquip, Gardena, California, USA) and identified by Ree's fauna key [9]. Because it was difficult to perform with all chiggers, 30 chiggers per small mammals were used.

DNA was extracted from 20 µl of chigger suspension using G-spin total DNA extraction kit (cat. no. 17046; Intron Biotechnology, Seoul, Korea). 200 µl of lysis buffer and 10 µl of proteinase K solution (20 mg/ml) was added to 20 µl of chigger suspension. The lysate was incubated at 56°C on a heating block for 30 min. After lysis, 200 µl of binding buffer was added. Then, the mixture was incubated at 70°C for 5 min. The mixture was applied to the spin column, and centrifuged at 13,000 rpm for 1 min. After discarding the filtrate, washing buffer A was added to the spin column and centrifuged at 13,000 rpm for 1 min. After discarding the flow-through, the

column was placed into a 2.0 ml collection tube. Washing buffer B was added to the spin column and centrifuged at 13,000 rpm for 1 min. After discarding the flow-through, placed column into a new 1.5 ml collection tube, and a total of 50 µl of elution buffer was added directly onto the membrane. After incubating for 1 min at room temperature, it was centrifuged for 1 min at 13,000 rpm to elute. DNA extract was stored at -20°C until amplification.

PCR was performed as INNOPLEX TSUTSU detection kit (cat. No. IPC10040; Intron Biotechnology). The kit was designed using primer sets to detect the 475 bp fragment gene encoding the 56 kDa antigen of *O. tsutsugamushi*. The first PCR was performed with 2 µl of DNA extract and 18 µl of distilled water treated with diethyl pyrocarbonate (DEPC; Gendepot, Barker, Texas, USA) in the first PCR premix tube. PCR conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycle at 94°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec; and final elongation at 72°C for 5 min using a Geneamp 9700 Biosystem (ABI, Foster City, California, USA). The second PCR was performed with 2 µl of the first PCR product and 18 µl of distilled water with DEPC in the second PCR premix tube. PCR conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycle at 94°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec, and final elongation at 72°C for 5 min using a Geneamp 9700 Biosystem (ABI). The final PCR products were evaluated by 1.5% agarose gel electrophoresis containing ethidium bromide.

The nucleotide sequence homology was aligned with sequence of previously published *O. tsutsugamushi* in Genbank by the BIOEDIT software program. The phylogenetic tree was generated by the neighbor-joining method (MEGA 6.0).

RESULTS

Small mammal collection

A total of 172 small mammals were collected. *Apodemus agrarius* was the most frequently collected 158 (91.8%), followed by *Myodes regulus* 8 (4.6%) and *Crocidura lasiura* 6 (3.5%). Overall, monthly mean trap rate was 14.3% (range 7-26%) (Table 1). For all periods, the trap rate for *A. agrarius* was higher for fallow ground (34.2%), followed by around water (29.1%), a boundary between forest and field (17.7%), around a tomb (13.9%), and a ridge between grain fields (5.1%) (Table 2).

Table 1. Number of small mammals captured by species (trap rate) in Gwangju CityMA, from September 2014 to August 2015

Month	No. traps	Trapping rate (%)	No. of small mammals			
			<i>Apodemus agrarius</i>	<i>Myodes regulus</i>	<i>Crocidura lasiura</i>	Total (%)
2014. 9	100	10	10	0	0	10 (10.0)
10	100	12	12	0	0	12 (12.0)
11	100	23	22	0	1	23 (23.0)
12	100	7	5	1	1	7 (7.0)
2015. 1	100	18	14	3	1	18 (18.0)
2	100	8	7	1	0	8 (8.0)
3	100	11	10	1	0	11 (11.0)
4	100	10	9	1	0	10 (10.0)
5	100	26	22	1	3	26 (26.0)
6	100	16	16	0	0	16 (16.0)
7	100	18	18	0	0	18 (18.0)
8	100	13	13	0	0	13 (13.0)
Total (%)	1,200	14.3	158 (91.8)	8 (4.6)	6 (3.5)	172 (100)

Table 2. Number (%) of small mammals captured by selected area in Gwangju MA, from September 2014 to August 2015

Species	No. of small mammals (%)					Total (%)
	Fallow ground	A ridge between rice fields	Boundary between forest and field	Around tomb	Around water	
<i>Apodemus agrarius</i>	54 (34.2)	8 (5.1)	28 (17.7)	22 (13.9)	46 (29.1)	158
<i>Myodes regulus</i>	0	0	4 (44.4)	4 (44.4)	1 (11.1)	9
<i>Crocidura lasiura</i>	1 (20)	0	1 (20)	2 (40)	1 (20)	5
Total (%)	55 (31.9)	8 (4.7)	33 (19.19)	28 (16.28)	48 (27.9)	172

Table 3. Number (%) of small mammals seropositive for *O. tsutsugamushi*, hantaviruses, *Rickettsia* spp., and leptospirosis that were captured in Gwangju MA, from September 2014 to August 2015

Species	No. small mammals tested	<i>O. tsutsugamushi</i>	Hanta-viruses	<i>Leptospira</i> spp.	<i>Rickettsia</i> spp.	Mixed infection				
						<i>O. tsutsugamushi</i> /hanta-viruses	<i>O. tsutsugamushi</i> / <i>Rickettsia</i> spp.	Hanta-viruses/ <i>Leptospira</i> spp.	Hanta-viruses/ <i>Rickettsia</i> spp.	<i>O. tsutsugamushi</i> /Hantaviruses/ <i>Rickettsia</i> spp.
<i>Apodemus agrarius</i>	145	37 (25.5)	23 (15.9)	2 (1.4)	22 (15.2)	9 (6.2)	7 (4.8)	1 (0.7)	3 (2.1)	1 (0.7)
<i>Myodes regulus</i>	7	4 (57.1)	1 (14.3)	0	0	0	0	0	0	0
<i>Crocidura lasiura</i>	4	0	0	0	0	0	0	0	0	0
Total (%)	156	41 (26.3)	24 (15.4)	2 (1.3)	22 (14.1)	9 (5.8)	7 (4.5)	1 (0.6)	3 (1.9)	1 (0.6)

Seropositive rates for *O. tsutsugamushi*, hantaviruses, *Rickettsia* spp., and *Leptospira* spp. in small mammals

Blood samples were obtained from 156/172 (90.7%). A total of 41/156 (26.3%) were seropositive for *O. tsutsugamushi* followed by small mammals positive for hantaviruses (24/156, 15.4%), *Rickettsia* spp. (22/156, 16.7%), and *Leptospira* spp. (2/172, 1.3%). Especially, tested 12 rodents were all positive for *O. tsutsugamushi* in October 2014. The prevalence for 4 vector-borne (deletion) infectious diseases was confirmed all the year round in Gwangju MA. Total 21 cases of mixed infections

were confirmed in *A. agrarius* (Table 3).

Seasonal distribution of chiggers and detection of *O. tsutsugamushi* by PCR

A total of 2,772 chiggers were collected from 158 *A. agrarius* (Table 4). *L. scutellare* 579 (54.1%) was the most commonly collected chiggers, followed by *L. orientale* 281 (1.8%), *E. koreanaensis* 94 (8.8%), *L. palpale* 46 (4.3%), *L. pallidum* 36 (3.3%), and *L. zetum* 7 (0.7%). The majority of *L. scutellare* were collected during the autumn (September-November, mean 5.6-

Table 4. Number (mean number) of chigger mites collected from *Apodemus agrarius* in Gwangju MA, between September 2014 and August 2015

Month	<i>L. pallidum</i>	<i>L. scutellare</i>	<i>L. palpale</i>	<i>L. orientale</i>	<i>L. zetum</i>	<i>N. gardellai</i>	<i>E. koreaensis</i>	Total	Captured <i>A. agrarius</i>	Collected chigger (%)
2014. 9	0	56 (5.6)	0	2 (0.2)	1 (0.1)	27 (2.7)	68 (6.8)	154 (15.4)	10	331 (11.9)
10	12 (1.0)	257 (21.4)	0	2 (1.7)	0	0	7 (0.6)	278 (23.2)	12	1,363 (49.2)
11	17 (0.7)	265 (12.0)	15 (0.7)	70 (3.2)	0	1 (<0.1)	12 (0.5)	380 (17.3)	22	755 (27.2)
12	0	0	0	0	0	0	0	0	5	0
2015. 1	1 (<0.1)	1 (<0.1)	25 (1.8)	50 (3.6)	3 (0.2)	0	1 (<0.1)	81 (5.8)	14	99 (3.6)
2	0	0	6 (0.9)	23 (3.3)	2 (0.3)	0	0	31 (4.4)	7	36 (1.3)
3	5 (0.5)	0	0	18 (1.8)	1 (0.1)	0	0	24 (2.4)	10	26 (0.9)
4	0	0	0	39 (4.3)	0	0	0	39 (4.3)	9	52 (1.9)
5	1 (<0.1)	0	0	67 (3.0)	0	0	4 (0.2)	79 (3.6)	22	98 (3.5)
6	0	0	0	2 (<0.1)	0	0	0	2 (<0.1)	16	2 (<0.1)
7	0	0	0	0	0	0	0	0	18	0
8	0	0	0	8 (0.6)	0	0	2 (<0.1)	10 (0.6)	13	10 (0.4)
Total (%)	36 (0.2) (3.3)	579 (3.7) (54.1)	46 (0.3) (4.3)	281 (1.8) (26.2)	7 (<0.1) (0.7)	28 (0.2) (2.6)	94 (0.6) (8.8)	1,071 (6.8) (100)	158	2,772 (100)

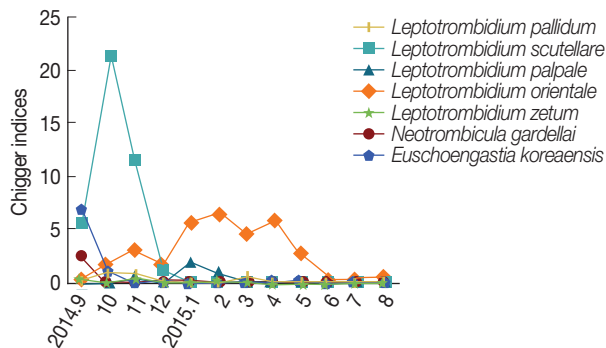


Fig. 2. Mean number of chigger mite species collected from *Apodemus agrarius* in Gwangju MA, from September 2014 to August 2015.

21.4), while *L. orientale* was collected in late autumn (October, mean 1.7) through spring (May, mean 3.0). A high number of *E. koreaensis* was collected in fall (September, 6.8), but infrequently during other months (Fig. 2).

The distribution of chiggers by species of small mammals was 7 chigger species on *A. agrarius*, 4 species on *M. regulus*, and no chiggers on *C. lasiura* (Table 5). A total of 3 chiggers (0.24%) were positive for *O. tsutsugamushi* (Table 5). Each sequence (1,410 mite-1 *L. scutellare*, 1,410 mite-2 *L. scutellare*, 1,504 mite-3 *L. orientale*) showed homology of 90.1%, 93.2%, and 98.2% with the Boryong strain (accession no. L04956) (Fig. 3). Two *L. scutellare* collected from *A. agrarius* (September 2014), and 1 *L. orientale* from *M. regulus* (April 2015) were

confirmed as positive for *O. tsutsugamushi*.

DISCUSSION

Seasonal serosurveillance of *O. tsutsugamushi*, hantaviruses, *Rickettsia* spp., and *Leptospira* spp. in small mammals and a study on chiggers collected from small mammals were conducted. For all periods, *A. agrarius* was the most frequently collected species (91.8%). Capture rates of *A. agrarius* were high for fallow ground area (34.2%) and around water (29.1%), places which were unmanaged and covered with 1 m cyperaceae. There were always several puddles in the fallow ground area, and this area was more likely to be damp than other areas. *C. lasiura* and *M. regulus* capture rates were high for the boundary between forest and field areas and the area around tombs, which were located in a hillier section than the fallow ground area. There were few grass bush in these areas. There were differences in species distribution by areas.

As shown in Table 3, the prevalence of scrub typhus was 25.5% in *A. agrarius*, lower than in surveys performed in Gyeonggi province, central part of ROK [10-12]. We confirmed evidence of infection of various zoonotic diseases in *A. agrarius* and observed evidence of scrub typhus and hantaviruses in *M. regulus*. Evidence of antibodies to hantaviruses and *Rickettsia* spp. implies that Gwangju MA area is not free of other zoonotic diseases.

In chigger mite’s distribution, *L. scutellare* was the predomi-

Table 5. Numbers of tested chigger mites (no. positive) assayed for *O. tsutsugamushi* by PCR in Gwangju MA, from September 2014 to August 2015

Species	<i>L. pallidum</i>	<i>L. scutellare</i>	<i>L. palpale</i>	<i>L. orientale</i>	<i>L. zetum</i>	<i>N. gardellai</i>	<i>E. koreaensis</i>	Total	Infection rate	Test rodent
<i>Apodemus agrarius</i>	36	579 (2)	46	281	7	28	94	1,071 (2)	0.19	158
<i>Myodes regulus</i>	0	6	12	141 (1)	1	0	5	165 (1)	0.59	8
<i>Crocidura lasiura</i>	0	0	0	0	0	0	0	0	0	6
Total	36	585 (2)	58	422 (1)	8	28	99	1,236 (3)	0.24	172

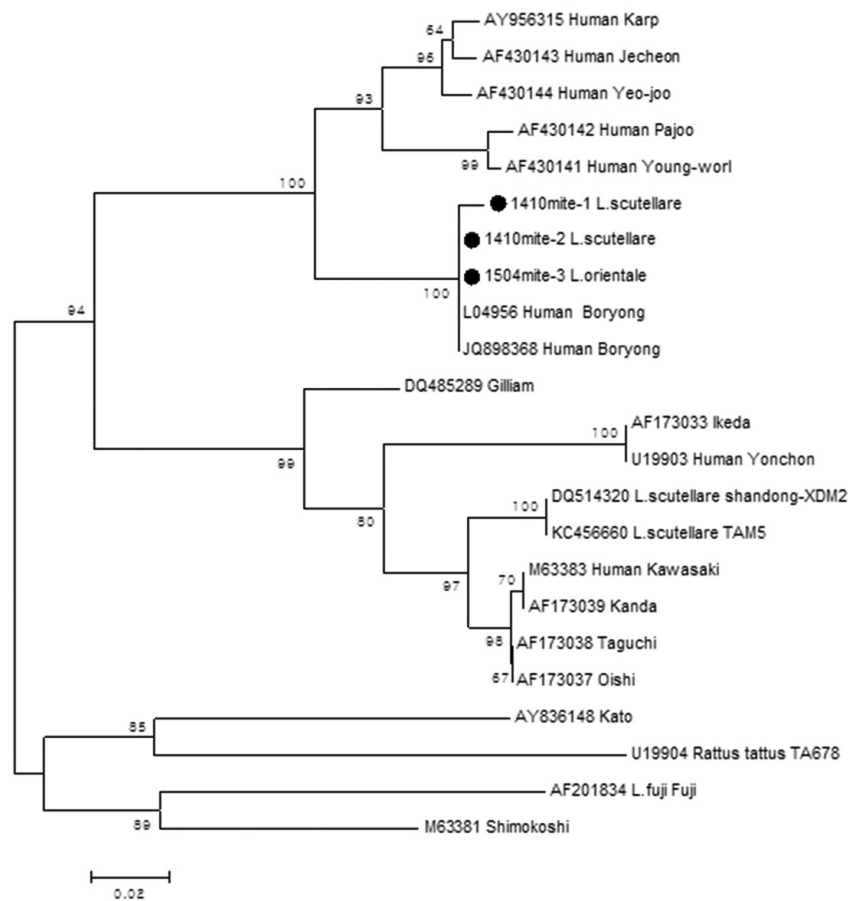


Fig. 3. Phylogenetic tree based on the nucleotide sequences of *Orientia tsutsugamushi* 56 kDa genes. The Neighbor-joining tree was constructed under kimura-2 parameter model (bootstrap 1,000 replicate) using MEGA 6.0. Gwangju metropolitan area strains (1,410 mite-2 *L. scutellare*, 1,410 mite-1 *L. scutellare*, 1,504 mite-3 *L. orientale*) identified are marked with filled circle.

nant chigger from *A. agrarius* in Gwangju MA and was similar to the results at Chonnam province [13-15]. Meanwhile, *L. pallidum* was the predominant species in central parts of ROK [16-18]. As shown in Fig. 2, there was seasonal difference of chigger mite species collected from *A. agrarius*. *L. scutellare* was highest from October through November 2014, and high populations of *L. scutellare* may contribute to human transmission

of scrub typhus in Gwangju MA area. *L. orientale* was highest in winter-spring seasons. This result indicates that the species of chigger mites could be affected by temperature and humidity.

Strains of scrub typhus show geographical variation. In ROK, the Boryong strain is known to be the predominant type in vectors and humans [3]. Our results showed that the 3 chiggers positive for scrub typhus were all Boryong strain. In our

study, it was found that small mammals were the source of various zoonotic diseases, and *L. scutellare* contributed to high chigger indices during the fall period. To reduce risk of zoonotic disease in Gwangju MA, it is necessary to clear fallow ground near humans' living space (cutting grass, filling puddles) for rodent control, and to provide adequate protection to farm workers (long sleeve clothes, boots). More information must be relayed about the risk of vector borne zoonotic diseases. Because information from 2 sites was limited, more data are needed over years regarding the relationship of disease transmission on humans, additional site selection, and distribution of gravid small mammals. In the future, our data will be helpful to provide prevention strategies against the resurgence of vector borne zoonotic diseases.

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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