MOLECULAR AND SEROLOGIC SURVEY OF ORIENTIA TSUTSUGAMUSHI INFECTION AMONG FIELD RODENTS IN SOUTHERN CHOLLA PROVINCE, KOREA

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Abstract. Field rodents were collected from six areas in southern Cholla Province, Korea from October to December 1993. Twenty-eight (24%) of the 119 Apodemus agrarius were seropositive (>1:10) for Orientia tsutsugamushi by the passive hemagglutination assay (PHA). Of the seropositive cases, 11 specimens had antibody titers greater than 1:80. No seropositive specimens were found among the eight Crocidura lasiura collected. On the other hand, the polymerase chain reaction (PCR) amplified about 520 basepairs of a gene encoding the 56-kD protein from the genomic DNA of 12 strains of O. tsutsugamushi tested. This target DNA sequence was amplified from the 11 (8.7%) blood specimens of A. agrarius, and one of the eight C. lasiura also showed evidence of O. tsutsugamushi infection by PCR. Only one of the PCR-positive samples was also PHA-positive. These results suggest that the PCR combined with a serologic assay more accurately detects the degree of infection of rodents with rickettsiae-causing scrub typhus in epidemiologic surveys.

Scrub typhus (tsutsugamushi disease) is caused by Orientia tsutsugamushi and is one of the most prevalent febrile illness in Korea, particularly from October through December. The first patient with tsutsugamushi disease in Korea was reported in 1951 by Munro-Faure and others, but no other cases were reported until 1985. In 1986, Lee and others and Yi reported tsutsugamushi disease among Korean residents. Chang and Kang isolated two strains of O. tsutsugamushi from Korean patients in 1986. Since then, tsutsugamushi disease has been found to be prevalent in all areas of Korea. The results of nationwide seroepidemiologic and microbiologic surveys conducted from 1986 to 1993 revealed that the O. tsutsugamushi seropositive rate among patients with acute febrile illness during these years varied from 27.7% to 51%. Scrub typhus occurs in all regions of Korea, including Cheju island. Seroprevalence in humans was found to be highest in south Kyungsang Province (21.2%), followed by south Cholla (14.6%), south Chungcheong (13.0%), north Kyungsang (12.1%), Kyunggi (11.5%), and least prevalent in north Cholla Province (10.6%).

Many animals have been implicated as the natural reservoirs of scrub typhus because they serve as hosts for chiggers of the vector species. Several wild mammalian species, such as Apodemus agrarius, Microtus fortis, Rattus norvegicus, and bats, are infected by O. tsutsugamushi. A serologic survey in rodents performed in 1986–1987 by Lee and others recorded the seropositive rates of the house mouse (5%), A. agrarius (58.9%), and Microtus spp. (77.7%). The results of this survey helped to explain the high prevalence rate of tsutsugamushi disease in Korea.

Although the infection rate among field rodents in central and northern Korea have been reported, seroepidemiologic studies on field rodents in southern Korea have not been done. Here, we report our investigations on the infection status of field rodents in southern Korea. The polymerase chain reaction (PCR) was also used to determine whether rodents that were negative for antibody by the passive hemagglutination assay were actually infected with O. tsutsugamushi.

MATERIALS AND METHODS

Field rodent collection and serum harvest. Field rodents were collected using traps placed in six areas (Changhung, Posong, Hwasun, Koksong, Hampyong, and Tamyang) in southern Cholla Province from October to December, 1993 (Table 1). The traps were set in the late afternoon and collected early the next morning. Live rodents were transported to the laboratory, where they were identified by species. Blood specimens were collected for the detection of antibodies and rickettsial DNA. The serum was prepared as described previously and stored at −70°C until use.

Cultivation of O. tsutsugamushi. The O. tsutsugamushi strains were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The Boryong and Yonchon strains were isolated from a patient as described previously. The other strains of O. tsutsugamushi were generously provided by Dr. A. Tamura (Niigata College of Pharmacy, Niigata, Japan). All of the strains of O. tsutsugamushi were grown in L929 cells (ATCC) as described previously. When the plaques were at a maximum, infected L cells were centrifuged at 18,000 × g for 20 min at 4°C. The pellets were washed three times and suspended in 200 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0).

Passive hemagglutination assay (PHA). Sheep red blood cells (sRBCs) were coated with a mixture of recombinant 56-kD protein derived from the Gilliam, Karp, and Boryong strains (1:1:1 [v/v/v]) as described previously. Briefly, sRBCs were treated with 0.002% tannic acid and 0.4% glutaraldehyde. The sRBCs were then suspended in phosphate-buffered saline (PBS) to 4% (v/v) and coupled to the recombinant antigen (10 µg/ml). The recombinant protein was purified as described previously. In our previous report, we described the serotypes of 113 strains isolated in Korea. The Boryong, Gilliam, and Karp strains were isolated from 77%, 11%, and 7% of the patients studied, respectively. We therefore used these three strains for all of the procedures in this study. The sensitized sRBCs were suspended in a diluent buffer (PBS, pH 7.2, 0.5% rabbit sera, 0.002% Tween 20)
to a final concentration of 0.6% (v/v). One part diluted serum specimen from each field rodent was mixed with one part sensitized sRBCs suspension in a U-bottom, 96-well, microliter plate in a final volume of 50 μl. In each plate, non-sensitized sRBCs and pre-immune mouse sera were used as negative controls. The reaction between the sera and sensitized sRBCs was allowed to occur at room temperature for 3 hr. The PHA titer was the final serum dilution that showed hemagglutination when mixed with sensitized cells.

**Polymerase chain reaction (PCR).** The part of the gene encoding the 56-kD protein was amplified from the genomic DNA of *O. tsutsugamushi* to assess the specificity of the PCR system. Genomic DNA from *O. tsutsugamushi* and *R. typhi*, as well as L929 cells and Vero cells (ATCC), infected with Hantaan virus was isolated as described previously. One hundred nanograms of these preparations were used as template DNA. One pair of the oligonucleotide primer was constructed to flank the hypervariable segment from the 253rd nucleotide to the 772nd nucleotide of the open reading frame that encodes the 56-kD protein of the Boryong strain (forward primer [TS1F], 5'-CCA GGA TTT AGA GCA GAG-3'; reverse primer [TS1R], 5'-CGC TAG GTT TAT TAG TAG CAT-3'). Oligonucleotide primers were synthesized and obtained from Oligo Etc., Inc. (Wilsonville, OR). The sequences of these primers were completely homologous with that of the gene encoding the 56-kD protein from the Boryong, Yonchon, Gilliam, Karp, and Kato strains (Figure 1). Between one and four basepairs were mismatched with the nucleotide sequence of the gene from other strains of *O. tsutsugamushi* as shown in Figure 1. The PCR was performed using the GeneAmp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT) in a total volume of 10 μl. Amplification was performed for 35 cycles, with denaturation for 15 sec at 94°C, annealing and extension for 20 sec at 60°C, and a final additional extension for 300 sec at 72°C. The presence and size of the amplified products were evaluated by 2% agarose gel electrophoresis in the presence of ethidium bromide.

**RESULTS**

**Collection of field rodents.** Table 1 summarizes the trapping rate of the field rodents and shows the species of the live rodents examined. Species within the genus *Apodemus*...
FIELD RODENTS INFECTED BY O. TSUTSUGAMUSHI IN KOREA

Table 2
Infection of Orientia tsutsugamushi among field rodents identified by passive hemagglutination assay (PHA) and polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Area</th>
<th>Test</th>
<th>PHA (&gt;1:10)</th>
<th>PHA (&gt;1:80)</th>
<th>PCR</th>
<th>PHA (+) and PCR (+)</th>
<th>Infection (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changhung</td>
<td>8/0</td>
<td>1/0</td>
<td>0/0</td>
<td>1/0</td>
<td>0/0</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Posong</td>
<td>6/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hwasun</td>
<td>28/2</td>
<td>5/0</td>
<td>4/0</td>
<td>0/0</td>
<td>0/0</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Koksong</td>
<td>34/1</td>
<td>5/0</td>
<td>1/0</td>
<td>1/1</td>
<td>0/0</td>
<td>8 (22.9)</td>
</tr>
<tr>
<td>Hampyong</td>
<td>25/0</td>
<td>7/0</td>
<td>0/0</td>
<td>1/1</td>
<td>0/0</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>Tamyang</td>
<td>25/0</td>
<td>7/0</td>
<td>0/0</td>
<td>1/1</td>
<td>0/0</td>
<td>6 (24)</td>
</tr>
<tr>
<td>Total (%)†</td>
<td>119/8</td>
<td>28/0 (22)</td>
<td>11/0 (8.7)</td>
<td>10/1</td>
<td>6/0 (0.8)</td>
<td>21 (16.5)</td>
</tr>
</tbody>
</table>

* Total number of rodents with a PHA titer greater than 1:80 or PCR-positive. Values in parentheses are percentages.
† Total number of rodents. Values in parentheses are percentages.

were the most predominant. The number of rodents and the rate of their collection in the respective sites was recorded because the density of the rodent population in the area could influence our data. From the 142 field rodents captured, 127 blood specimens (119 from A. agrarius and eight from C. lasiura) were used in this study.

Antibodies in the field rodents. Sera from the rodents were examined for anti-O. tsutsugamushi antibodies by PHA. The antibody detection rates among these serum specimens are shown in Table 2. Positive serum samples had titers ranging from 1:10 to 1:320 (Figure 2). Antibody titers greater than 1:10 were observed in 22% of the A. agrarius specimens tested. Eleven (9.2%) of the 119 serum specimens from A. agrarius showed antibody titers greater than 1:80 by PHA. These serum specimens were obtained from Hwasun, Koksong, and Tamyang. None of the rodents with serum antibody titers greater than 1:80 by PHA were collected in Changhung or Hampyong. The PHA-positive (> 1:10) rates and antibody titers varied according to the area from which the rodents were captured (Figure 2.). Sera from rodents collected in Koksong showed the highest positive rate. Antibody to O. tsutsugamushi was not detected in all serum specimens from any of the eight C. lasiura collected.

Detection of O. tsutsugamushi DNA by PCR. The primer sequences were completely or almost completely homologous to the nucleotide sequences of the gene that encodes the 56-kD protein from various strains (Figure 1). The target DNAs were amplified from all of the strains tested (Figure 3). The TS1F-TS1R primer pairs amplified a 520-basepair fragment from the Boryong strain. This fragment size corresponds to the nucleotide length predicted by the DNA sequence. No specific amplification was observed from the genomic DNA of L929 cells, R. typhi, or Vero cells infected with Hantaan virus.

To determine the sensitivity of the PCR, the part of the gene that encodes the 56-kD protein was amplified from plasmid pMBR56 DNA.19 This DNA was serially diluted in distilled water and used as a target DNA. The PCR products were electrophoresed and hybridized with digoxigenin-labeled pMBR56 plasmid DNA. Ten attograms of pMBR56 DNA, which corresponds to three copies of the target DNA, could be detected by Southern blot analysis. The DNA fragments were amplified from the blood specimens of field rodents (Figure 4). The pMBR56 plasmid DNA19 served as a positive control, and 10% mouse blood in distilled water served as a negative control.

As shown in Table 2, rickettsial DNA was detected by PCR from 8.7% of the field rodent specimens. The detection rate was 8.4% in A. agrarius and 12.5% in C. lasiura. Rickettsial DNA was detected in one of five C. lasiura captured in Hampyong. The serum specimen from one A. agrarius captured in Koksong gave positive results by both PHA (1:80) and PCR.

If the PCR- or PHA-positive reactions (> 1:80) were considered as evidence of past or recent infection by O. tsutsugamushi, 16.5% of the field rodents showed evidence of infection by O. tsutsugamushi. The highest infection rate was demonstrated in Koksong. The field rodents captured in

![Figure 2](https://example.com/figure2.png)
Posong did not show any evidence of rickettsial infection (Figure 5).

DISCUSSION

To obtain basic but essential epidemiologic information about scrub typhus in southern Cholla Province, where the tsutsugamushi disease is endemic, we examined blood specimens from local field rodents for O. tsutsugamushi DNA and antibodies to the organism. The rodents were captured during October through December, when the tsutsugamushi disease is most prevalent in this area. The trapping rate was higher than that reported for studies performed with field rodents collected in Kyounggi and Kangwon Provinces (7.8%). This difference suggests that the density of the rodent population in this area is higher than that in other areas. This higher density of field rodents increases the likelihood of human bites by mites and helps to explain the high endemic rate of tsutsugamushi disease in this area. Sixty-nine percent of the field rodents collected in this area were parasitized by chiggers, Leptotrombidium pallidum, one of the vector species of tsutsugamushi disease, was the dominant species. The chigger index of the rodents collected in Hwasun and Koksong was higher than that of the other areas. The rodents collected here also showed higher infection rates, suggesting that the infection rate of rodents by O. tsutsugamushi correlates well with the infestation rate by chiggers.

In our previous report, eight serum specimens from healthy individuals showed PHA titers of 1:40, and one serum specimen showed a titer of 1:80. The sera with PHA titers greater than 1:80 were considered PHA-positive whereas the others were considered PHA-negative in that study. Because the criteria for antibody titers used in hu-
FIELD RODENTS INFECTED BY *O. TSUTSUGAMUSHI* IN KOREA

7. Chang WH, 1988. Occurrence of tsutsugamushi disease and pro-


