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 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, Koskong, 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 Gilliam, Karp, and Kato strains were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The Boryong, Gilliam, and Karp strains were isolated from a patient as described previously. 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)
TABLE 1
Collection of the field rodents at southern Cholla Province, Korea from October through December 1993

<table>
<thead>
<tr>
<th>Area</th>
<th>No. of traps set</th>
<th>No. of rodents collected</th>
<th>Trapping rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changhung</td>
<td>90 8 1 9 10</td>
<td>Changhung</td>
<td>90 8 1 9 10</td>
</tr>
<tr>
<td>Posong</td>
<td>90 8 1 9 10</td>
<td>Posong</td>
<td>90 8 1 9 10</td>
</tr>
<tr>
<td>Hwasun</td>
<td>162 30 3 33 20.4</td>
<td>Hwasun</td>
<td>162 30 3 33 20.4</td>
</tr>
<tr>
<td>Koksong</td>
<td>153 34 1 35 22.9</td>
<td>Koksong</td>
<td>153 34 1 35 22.9</td>
</tr>
<tr>
<td>Hampyong</td>
<td>156 22 5 27 17.3</td>
<td>Hampyong</td>
<td>156 22 5 27 17.3</td>
</tr>
<tr>
<td>Tamyang</td>
<td>163 29 0 29 17.8</td>
<td>Tamyang</td>
<td>163 29 0 29 17.8</td>
</tr>
<tr>
<td>Total (%)</td>
<td>814 131 (92.3) 11 (7.7) 142 (100) 17.4</td>
<td>Total (%)</td>
<td>814 131 (92.3) 11 (7.7) 142 (100) 17.4</td>
</tr>
</tbody>
</table>

* Trapping rate = (no. of rodents collected/no. of traps set) × 100.

FIGURE 1. Alignment of primer sequences with nucleotide sequences of the 56-kD protein gene from 12 strains of Orientia tsutsugamushi. Conserved nucleotides are marked by dots.

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, microtiter plate in a final volume of 50 µl. In each plate, nonsensitized 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 L.929 cells and Vero cells (ATCC), infected with Hantaan virus was isolated as described previously.20 One hundred nanograms of these preparations were used as template DNA. The DNA of the plasmid pMBR56 19 was serially diluted in distilled water and used as a target DNA for the PCR to determine its sensitivity. For Southern blot analysis of the amplified products, digoxigenin labeling of the probe, capillary blotting, and hybridization were carried out using a DIG DNA Labeling and Detection Kit® as described by the manufacturer (Boehringer Mannheim Corp., Indianapolis, IN).

The whole blood specimens, which were stored at −70°C, were frozen and thawed twice and then diluted 10-fold with distilled water. A diluted specimen (1 µl) was 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...
Infection of Orientia tsutsugamushi among field rodents identified by passive hemagglutination assay (PHA) and polymerase chain reaction (PCR)

Table 2

<table>
<thead>
<tr>
<th>Area</th>
<th>Test</th>
<th>PHA (+)</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>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>
</tr>
<tr>
<td>Hwasun</td>
<td>28/2</td>
<td>5/0</td>
<td>4/0</td>
<td>0/0</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Koksong</td>
<td>34/1</td>
<td>3/0</td>
<td>0/0</td>
<td>5/0</td>
<td>8 (22.9)</td>
</tr>
<tr>
<td>Hampyong</td>
<td>18/5</td>
<td>5/0</td>
<td>0/0</td>
<td>1/1</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>Tamyang</td>
<td>25/0</td>
<td>7/0</td>
<td>3/0</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 (8.7)</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. 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 DNA 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

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**Figure 2.** Highest antibody titers to Orientia tsutsugamushi by passive hemagglutination assay (PHA). Each circle represents one serum specimen. The plot indicates the highest titer by the PHA against the 56-kD protein of the O. tsutsugamushi Gilliam, Karp, and Boryong strains. To elucidate the data for each sample, the overlapping points were plotted separately by using the area variable.
FIGURE 3. Agarose gel electrophoresis of DNA fragments from the chromosome of Orientia tsutsugamushi amplified by the polymerase chain reaction. The hypervariable region of the gene encoding the 56-kD protein was amplified. Lane 1, Gilliam; lane 2, Karp; lane 3, Kato; lane 4, Boryong; lane 5, Kawasaki; lane 6, Shimokoshi; lane 7, TA678; lane 8, TA686; lane 9, TA716; lane 10, TA763; Lane 11, TH1817; lane 12, Yonchon; lane 13, L929. The nucleotide length of the amplified fragment from bor56 was 520 basepairs (bp) and is indicated on the figure. The molecular size marker, Hind III-digested phage lambda DNA, is also shown (lanes M). The sizes of the marker DNAs are 23, 130, 9,416, 6,557, 4,316, 2,322, 2,027, and 564 bp.

FIGURE 4. Agarose gel electrophoresis of DNA fragments from blood specimens of field rodents amplified by the polymerase chain reaction (lanes 1–11). Lane 1, Changhung; lanes 2–6, Koksong; lanes 7 and 8, Hapmyong; lanes 9–11, Tamyang. The amplified product in lane 8 is from the blood of Crocidura laziura and the others are from Apodemus agrarius. Plasmid pMBR56 DNA was used as a positive control (lane P), and 10% mouse blood in distilled water was used as a negative control (lane N). The molecular size marker, Hae III-digested phage λX174 phage DNA, is also shown (lane M). The sizes of the marker DNAs are 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 basepairs. The value on the left is in basepairs.

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. TSUTSGAMUSHI IN KOREA

Figure 5. Geographic distribution of the field rodents captured in southern Cholla Province of Korea. The percentage of rodents that showed evidence Orientia tsutsugamushi infection is shown. The numbers in parentheses denote the percentage of rodents that showed evidence of infection in each area.

man studies cannot be directly applied to studies of wild rodents, reasonable criteria appropriate to rodent studies should be determined. Therefore, we assigned titers > 1:80 as indicator of previous exposure to O. tsutsugamushi.

Seventeen percent of the field rodents in southern Cholla Province showed evidence of infection with O. tsutsugamushi, and infected rodents were observed in all of the investigated areas except Posong. The seropositive rate of scrub typhus among patients with acute febrile episodes was highest (53.7%) in southern Cholla Province in 1993. In addition, the epidemiologic survey carried out from 1986 to 1992 showed that the new cases of scrub typhus in southern Cholia Province was as high as 14.0% of patients with acute febrile episode. Given the high infection rate of field rodents found in this study and the high occurrence of patients in this area, these two factors are thought to be significantly correlated.

Although O. tsutsugamushi infection has always been associated with the presence of anti-rickettsia antibodies in wild rodents, the presence of antibodies in these animals does not always indicate active infection. In other study, the organism could not be isolated from most of the rodents that were antibody positive at the time of capture. Orientia tsutsugamushi could be isolated from rodents as early as four days after its inoculation, but antibodies did not appear until day 11. The antibody in serum could be used as a marker for past or recent infection by O. tsutsugamushi; however, the lower infection rate could be estimated by the serologic assay alone because the antibody titers are not yet high enough to be detected at the early phase of infection. Rates of rickettsial infections could be determined more accurately by testing for the antibody and by screening for O. tsutsugamushi DNA.

Orientia tsutsugamushi usually replicates in mammalian endothelial and mononuclear cells. The splenocytes of infected mammals have been widely used to detect organisms. In our previous report, we showed that O. tsutsugamushi DNA could be detected in the blood 89 days after infection. However, the DNA could not be amplified further from the spleen of mice 51 days after the infection. Furthermore, the target sequences in the spleen were amplified less efficiently than those in blood cells. For these reasons, we used blood rather than splenocytes in our PCR analysis. Some factors in blood are known to inhibit the PCR; however, we did not detect any inhibitory effect on our assay after dilution of the samples (> 1:10). The sensitivity of our PCR analysis could be increased using whole blood without conventional DNA extraction since part of the template DNA could be lost during the extraction procedures.

The target DNA from all of the strains of O. tsutsugamushi were amplified by the PCR system used in this study. This finding suggests that the gene encoding the 54–56-kD protein of O. tsutsugamushi could be used as a target in the molecular epidemiology of scrub typhus. In addition, we believe that the serologic method combined with the PCR offers more accurate epidemiologic analysis than using either method alone.

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