Development of a Recombinant Protein-Based Enzyme-Linked Immunosorbent Assay and Its Applications in Field Surveillance of Rodent Mice for Presence of Immunoglobulin G against Orientia tsutsugamushi

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A recombinant protein containing the immunodominant conserved epitope region of the 56-kDa outer membrane protein of the Karp strain of Orientia tsutsugamushi was purified to near homogeneity using recombinant DNA techniques. The purified protein was used to immunize rabbits and produced an antibody that could recognize different strains of O. tsutsugamushi, as demonstrated both by Western blotting and immunofluorescence assay. An enzyme-linked immunosorbent assay (ELISA) based on this recombinant protein was developed to detect antibody (immunoglobulin G [IgG]) against O. tsutsugamushi in mice captured in different districts of Taiwan during 2000 to 2001. A significant difference was found in the antibody seroprevalence rates of Suncus murinus mice captured in different districts of Taiwan ($\chi^2 = 26.64; P < 0.05$). Furthermore, a significant difference of IgG seropositivity rates was observed among different kinds of mice ($\chi^2 = 93.85; P < 0.05$). Antibody seropositivity rates were higher in Bandicota indica (100%), Rattus flavipes (96.17%), and Rattus luteus (95.83%) than in Rattus norvegicus (86.05%) and Rattus mindanensis (83.67%) ($\chi^2$ = 12.59, $P < 0.05$). The lowest antibody seropositivity rate (54.4%) was observed in Suncus murinus. Antibody seropositivity rates of mice from different districts differed significantly because of the significant difference in antibody seroprevalence rates for S. murinus. The results of this study indicated that the recombinant protein ELISA developed in this study could be used to conduct large-scale surveillance of rodent mice for the presence of antibody against O. tsutsugamushi. The high seroprevalence rates in rodent mice (except S. murinus) suggest that people residing in these districts are at increased risk of developing O. tsutsugamushi infection.

Scrub typhus is an acute febrile disease endemic in Asia-Pacific regions including Korea, Japan, China, the Philippines, Thailand, and Taiwan (8, 25, 26, 28, 29, 32, 33, 35). The causative agent, Orientia (formerly Rickettsia) tsutsugamushi, is a gram-negative obligate intracellular bacterium which has been isolated from a variety of eukaryotic host cells and is transmitted via chiggers. O. tsutsugamushi causes local inflammation accompanying eschars at the site of infection, which then spreads systemically (6). Orientia isolates are antigenically diverse, resulting in numerous serotypes. Several antigenic variants, such as representative strains Gilliam, Karp, Kato, Boryong, Shimokoshi, Kawasaki, Kuroki, and other isolates have been reported (7, 8, 11, 22–24, 33, 36).

Traditionally, confirmatory diagnosis of scrub typhus is generally based on serologic procedures, such as the Weil-Felix test, the immunoperoxidase test, and the immunofluorescence (IF) test (4, 5). However, these serodiagnostic tests have shortcomings or requirements which limit their usefulness. A more practical approach to the diagnosis of scrub typhus is to detect the antibody using a specific and immunodominant protein of O. tsutsugamushi or to detect antigen using a specific antibody. The 56-kDa immunodominant protein of O. tsutsugamushi is reactive with group-specific and strain-specific monoclonal antibodies, suggesting the existence of group-specific and strain-specific epitopes in this molecule (30). The immunogenicity of this protein suggests that it is an a priori diagnostic antigen candidate. Several studies dealing with the antibody responses obtained by using this recombinant 56-kDa protein as bait in enzyme-linked immunosorbent assay (ELISA) have been reported (9, 13, 15). ELISA has been demonstrated to have, as demonstrated by Kim et al. (13) and Land et al. (15), high sensitivity and specificity for serodiagnosis of O. tsutsugamushi.

In recent years, both the reported and identified cases of scrub typhus have increased in various districts of Taiwan (9a). The reasons for this increase remain to be investigated. However, epidemiologic surveillance of rates of seroprevalence against O. tsutsugamushi among different kinds of rodent mice in different districts might be an appropriate first step in exploring the reasons responsible for the increasing frequency of reports of scrub typhus cases from Taiwan. In this study, an ELISA was developed using recombinant truncated proteins which contain the epitope region of the 56-kDa outer membrane protein of the Karp strain. This test was used to survey seroprevalence rates among different kinds of rodent mice against O. tsutsugamushi in different districts of Taiwan.

MATERIALS AND METHODS

Bacterial strains and vectors. Escherichia coli DH5α or HB101 was used for cloning, and E. coli BL21(DE3) was used for overexpression of proteins under...
the control of the phase T7 promoter. The plasmid vector pRSET-B (Invitrogen) was used for the cloning of an expression plasmid that expresses the histidine
tagged truncated protein rP56a.

Construction of plasmid expressing truncated 56-kDa outer membrane protein
tein. Plasmid DNA. Genomic DNA was extracted from L292 cells infected with the Karp strain of *O. tsutsugamushi* using the method described by Maniatis et al. (20) with some modifications. Extracted DNA resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0) was used as a template in the PCR for the amplification of amino acids (a.a.) 31 to 274 of the Karp strain p56-kDa outer membrane protein (Kp56a) gene of *O. tsutsugamushi*. A pair of primers corresponding to nucleotides 634 to 663 and nucleotides 1357 to 1377 (5′-EcoRI, 5′-GGGGGTACCCGCGACTTTGAGTGTTGTCCTTATGC-3′; 3′-EcoRI, 5′-GGGGAACTTCAATCATATGTTAGGAT-3′; where cutting sites are underlined and start and stop codons appear in boldface) of Karp strain 56-kDa-protein-encoding sequences were designed based on the published sequences (GenBank accession number M53004).

The restriction enzyme site for KpnI was incorporated at the 5′ terminus of the coding sequence ampiclon. An in-frame termination codon followed by an EcoRI re-
striction site was introduced at the 3′ terminus of the coding sequence ampiclon. The PCR products were carried out with an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 2 min), with a final elongation step of 72°C for 5 min. Each reaction buffer contained 5% dimethyl sulfoxide. The ampli-
fied coding sequence DNA was digested with KpnI and EcoRI enzymes, and the resulting 863-bp fragment (nucleotides 556 to 1419) of the coding sequence DNA was inserted into the KpnI and EcoRI sites of pSET8 vectors to form the plasmid pRSETB-Kp56a. Recombinant plasmid DNA was sequenced and con-

firmmed.

Preparation of bacterial competent cells. Stacked *E. coli* strains (DH5α or BL21(DE3)) were used to prepare competent cells according to the method of Ausubel et al. (1). Competent cells were aliquoted and stored at −70°C immediately.

Transformation of bacteria. Transformation of bacteria was performed on a
Luria-Bertani agar plate containing ampicillin (100 µg/mL) according to the method of Ausubel et al. (1).

Plasmid DNA preparations. Plasmid DNA used for cloning was prepared by
alkaline lysis and a precipitation, an equal volume of isopropanol was used and the mixture was allowed to

fermentation, the procedures of plasmid DNA preparation in this study were
the same as the procedures of references listed; however, in the DNA precipi-
tation, an equal volume of isopropanol was used and the mixture was allowed to sit for 5 min at room temperature. Plasmid DNA used to express recombinant protein or to be sequenced was prepared by large-scale alkaline lysis and a
polyethylene glycol (PEG) precipitation method (17, 18, 19).

Expression and purification of recombinant protein. For prokaryotic expres-
sion and purification of histidine-tagged proteins, *E. coli* strain BL21(DE3) containing plasmid pRSETB-Kp56a was used. The *E. coli* strain harboring this plasmid was grown to an optical density (OD) at 600 nm of 0.7 to 0.8 prior to induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (dissolved in 100% DMSO) at 37°C for 4 h. For induction (see Fig. 4), cells were harvested and lysed by resuspending the bac-
terial pellets in sonication buffer (containing 50 mM sodium phosphate [pH 8.0], 10 µM phenylmethylsulfonyl fluoride, 0.1% Tween 20, 100 mM KCl, 500 mM NaCl, and 1 mg of lysozyme per ml) for 30 min prior to sonication. Following centrifugation at 17,000 × *g* (4°C for 20 min) in a Beckman J2-MI centrifuge with a JA 25.5 rotor, the pellets were resuspended in buffer B (8 M urea, 0.1 M sodium phosphate [pH 8.0], and 10 mM Tris [pH 4.5]) and stirred at room temperature for 1 h. After centrifugation, the supernatant was purified via metal chelate chromatography using Ni²⁺-nitrilotriacetic acid (NTA) complexes (Qiagen). Briefly, 20 ml of supernatant was passed through a 2-ml column of Ni²⁺-NTA agarose that was prewashed with buffer B, buffer C (8 M urea, 0.1 M sodium phosphate, 10 mM Tris [pH 6.3]), and buffer F (6 M guanidine-HCl, 0.2 M acetic acid) and preequilibrated in buffer B. The column was then washed with 10 volumes of buffer B and then with 10 volumes of buffer C, and the protein was eluted with buffer D (8 M urea, 0.1 M sodium phosphate, 10 mM Tris [pH 5.9]) and buffer E (8 M urea, 0.1 M sodium phosphate, 10 mM Tris [pH 4.5]) in fractions of 0.5 ml. Protein-containing fractions were identified by sodium do-
decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. To renature the protein, a stepwise dialysis was performed at 4°C against buffer B containing decreasing concentrations of urea (4, 2, 1, 0.5, 0.25, 0.125, and 0.05 M) and against buffer D (20 mM HEPEs [pH 8.0], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, and 0.2% Nonidet P-40) alone. After dialysis and a short centrifugation step, the solution in a 15-ml centrifuge tube was loaded onto a Ni²⁺-NTA agarose that was prewashed with buffer B, buffer C (8 M urea, 0.1 M sodium phosphate, 10 mM Tris [pH 4.5]) and against buffer E (8 M urea, 0.1 M sodium phosphate, 10 mM Tris [pH 6.8]) containing 1/1,000 volume of 35% H₂O₂) was added and incubated at room temperature for 15 min. After the reaction was stopped by adding 1 M H₂SO₄ (50 µl/well), the ODs at 450 nm were measured. Sera were always assayed in duplicate. Each plate included an air blank, a negative control (preimmune serum in triplicate), as well as a row of different diluted positive controls used to establish a standard curve.

Cutoff value for ELISA. Backgound reactivity and possible cross-reactivity were assessed by analyzing preimmune serum specimens from healthy rabbits, mice, and rats. The cutoff values were set at OD₅₀ + 3 standard deviations, where OD₅₀ is the mean of OD₅₀ of the preimmune serum specimens. The OD₅₀ of these preimmune serum specimens varied from 0.064 to 0.131 for the rabbit anti-P56 IgG assay, from 0.05 to 0.06 for the mouse anti-P56 IgG assay, and from 0.046 to 0.053 for the rat anti-P56 IgG assay. In this way, for all the analyses described below, sera were classified as being above or below the OD threshold.

Western immunoblotting analysis. For the identification of purified rP56a truncated (Karp strain a.a. 31 to 274) protein, Western immunoblot analysis (16, 17) was performed. Briefly, the purified protein was boiled in a sample buffer (125 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 20% glycerol, 0.005% bromophenol blue) for 5 min and then loaded onto an SDS–12% polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon membrane (Millipore) by semidy methods (Hof-
fer). The membranes were blocked with BLOTTO-Tween blocking solution and then incubated with mouse anti-histidine antibody (1:1,000; Amersham Pharma-
cia Biotech). The membrane was then washed with blocking buffer and further incubated with HRP-conjugated secondary antibody. The membrane was finally washed with blocking buffer and developed with ECL Western blotting reagents (Amersham Pharmacia Biotech) or with DAB peroxidase substrate (Sigma Fast [catalog no. D-4418]; Sigma) (DAB [0.7 mg/ml] in 0.06 M Tris [pH 7.6] with H₂O₂ [0.17 mg/ml] and 0.1% NiCl₂.)

For the identification of rabbit, mouse, or rat anti-rP56a antisera, sera from immunized rabbits, mice, or rats were used as primary antibodies to react with purified rP56a protein in a Western blot assay. For the identification of expres-
sions of wild-type or truncated P56 protein, equal amounts (approximately 50 mg) of nuclear proteins from extracts of infected or transfected cells (mouse L929 or human Vero cells) were boiled in a sample buffer (125 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 20% glycerol, 0.005% bromophenol blue) for 5 min and then loaded onto an SDS–12% polyacrylamide gel. After electrophoresis, proteins were transferred to an Immobilon membrane (Millipore) by semidy methods (Hof-
fer). The membranes were blocked with BLOTTO-Tween blocking solution and then incubated with mouse anti-histidine antibody (1:1,000; Amersham Pharma-
cia Biotech). The membrane was then washed with blocking buffer and further incubated with HRP-conjugated secondary antibody. The membrane was finally washed with blocking buffer and developed with ECL Western blotting reagents (Amersham Pharmacia Biotech) or with DAB peroxidase substrate (Sigma Fast [catalog no. D-4418]; Sigma) (DAB [0.7 mg/ml] in 0.06 M Tris [pH 7.6] with H₂O₂ [0.17 mg/ml] and 0.1% NiCl₂.)

For the recognition capability of rabbit anti-rP56a, indirect IF assay was performed. Briefly, the mouse anti-rP56a was incubated with infected or noninfected cells with different strains of *O. tsutsugamushi* as described below, sera were classi-
dated as being above or below the OD threshold.
O. tsutsugamushi L929 cells. The cells infected with (from the Institute of Preventive Medicine, Nan Kong) and used to infect mouse fluorescence microscopy. and examined by immuno
d(10 min per washing) with phosphate buffer, pH 7.4, glass slides were mounted temperature for 30 min and 5 min, respectively. After being washed three times PBS, pH 7.4; catalog no. D 9542; Sigma) were added and incubated at room,6-diamidino-2-phenylindole) (1:1,000 in 1:100 in PBS, pH 7.4) and DAPI (4
/ H11032 IgG-FITC conjugate [catalog no. 112-095-008; Jackson Immuno Research],
jugate [catalog no. 115-015-008; Jackson Immuno Research] and goat anti-rat pteric acid corresponding to the conserved region of outer membrane protein among different strains of O. tsutsugamushi. A conserved sequence which contains the epitope region of the antigen and encodes the outer membrane proteins of different strains of O. tsutsugamushi was found in GenBank using the CLUSTAL W (version 1.8) multiple sequence alignment program. Arrows indicate the initiation and stop points of amino acids amplified by PCR. (B) Purification of recombinant p56 outer membrane protein (O. tsutsugamushi Karp strain a.a. 31 to 274). Histidine-tagged truncated rP56Δ expressed in E. coli BL21(DE3) was purified by nickel chelate chromatography as described in Materials and Methods. The protein was resolved by SDS–12% PAGE and stained with Coomassie blue. Lane 1, marker; lanes 2 and 3, total cell lysates from E. coli transformed with pRSETB vector (lane 2) and pRSETB-karp56 (a.a. 31 to 274) plasmid DNA (lane 3), respectively; lane 4, fractions washed with buffer B; lane 5, fractions washed with buffer C; lanes 6 and 7, fractions eluted with buffer D; lanes 8 and 9, fractions eluted with buffer E. The arrow indicates the predicted histidine-tagged p56 truncated protein. *, protein induction for 3 h by IPTG.

RESULTS

Purification of recombinant truncated outer membrane protein of O. tsutsugamushi. A conserved sequence, which contains the immunodominant epitope region (that encompassed ADI-ADII and part of ADIII) of the antigen (28), encoding the outer membrane proteins of different strains of O. tsutsugamushi was obtained from GenBank using the CLUSTAL W (version 1.8) multiple sequence alignment program (Fig. 1a). This coding sequence, corresponding to amino acids 31 to 274 of the P56 outer membrane protein of Karp strain, was amplified by PCR and cloned into the prokaryotic expression vector containing a six-histidine tag coding sequence, pRSET-B, to produce pRSETB-Kp56Δ plasmid. The pRSETB-Kp56Δ plasmid allows overexpression of the six-histidine-tagged truncated outer membrane protein in E. coli BL21(DE3), and the recombinant protein could be purified by nickel chromatography since the histidine region of the fusion protein will bind nickel ions. After sequencing, the expression plasmid was transferred into E. coli [strain BL21(DE3)], and the recombinant protein was induced by IPTG to confirm the correctness of expression. Recombinant protein was purified to near homogeneity using Ni2+-NTA agarose (Qiagen) affinity chromatography (Fig. 1b, lane 8) and was confirmed by Western blotting with mouse antihistidine monoclonal antibody (data not shown). Results from this study demonstrated that the purified
protein was a recombinant fusion protein containing the truncated outer membrane protein of *O. tsutsugamushi*.

**Antibody against recombinant outer membrane protein (rP56Δ) can recognize different strains of *O. tsutsugamushi*.** After immunization of the rabbit, the polyclonal antibody was obtained and confirmed to react with both recombinant rP56Δ protein and outer membrane protein of *O. tsutsugamushi* by Western blotting (Fig. 2). This antibody was further confirmed to recognize different strains of *O. tsutsugamushi* by IFA (Fig. 3). Antibody against *O. tsutsugamushi* was measured and confirmed to live-longed with a titer higher than 204,800 even at 6 months after the last immunization (Fig. 4). Furthermore, it was demonstrated that mice and rats immunized with this purified recombinant protein could also produce high titers of antibody (IgG) against the outer membrane protein (P56) of *O. tsutsugamushi* (data not shown). These anti-rP56Δ mouse and rat antibodies were used as positive controls and used to create a standard curve by which IgG titers of mouse and rat sera measured at different days by ELISA could be calculated and adjusted (data not shown).

**Antibody assays with recombinant protein ELISA obtain good reproducibility with traditional micro-IFA test using native *O. tsutsugamushi* antigen.** Results of mouse IgG against *O. tsutsugamushi* obtained by ELISA were comparable with the results obtained by traditional micro-IFA. Of a total of 144 random serum samples assayed for their IgG antibody reactions against *O. tsutsugamushi*, findings were consistent for 134 samples. The κ coefficient was 0.735 with a kappa statistic equal to 8.96 (P < 0.05) (Table 1). These results showed that the recombinant ELISA used in this study had good reproducibility based on comparison with the traditional micro-IFA test.

**Seroprevalence rates of IgG against *O. tsutsugamushi* differ significantly among different species of mice.** ELISA was used to evaluate the presence of antibody against *O. tsutsugamushi* in mice captured in different districts during 2000 and 2001. No significant differences were found in the antibody seroprevalence rates for the same strains of mice captured in different districts. However, different seroprevalence rates of IgG against *O. tsutsugamushi* among different strains of mice were observed (χ²5, 0.95 = 93.85; P < 0.05). Antibody seropositivity rates were higher in *Bandicota indica* (100%; 3 of 3), *Rattus flavivertex* (96.17%; 176 of 183), and *Rattus luteus* (95.83%; 23 of 24) than in *Rattus norvegicus* (86.05%; 74 of 86) and *Rattus mindanensis* (83.67%; 41 of 49) (χ²4, 0.95 = 12.59; P < 0.05). The lowest antibody seropositivity rate (54.4%; 68 of 125) was observed in *S. murinus* (Table 2). Significant differences in seroprevalence rates among different strains of mice suggested potential for involvement with the reported cases of scrub typhus in different districts. However, this implication requires further detailed study.

**Different compositions of mouse strains contributed to the differences in seroprevalence rates of mice against *O. tsutsugamushi* among different districts.** Significant differences were seen in antibody (IgG) seroprevalence rates of mice in different districts of Taiwan. Antibody seroprevalence rates of mice were generally greater in Penghu County (91.58%; 87 of 95), Kinmen County (84.82%; 218 of 257), and Orchid Island (83.67%; 41 of 49) than in Hwalien County (56.86%; 29 of 51) and Lienkiang County (55.56%; 10 of 18) (χ²4, 0.95 = 35.24; P < 0.05) (Table 2). However, significant differences in the antibody seroprevalence rates of mice among different districts were due to differences of proportional rates of *S. murinus* in the mouse populations (χ²3, 0.95 = 35.24; P < 0.05) (Table 2). Excluding *S. murinus* from the analysis, no significant differences in antibody seroprevalence rates were found for mice captured from different districts (χ²2, 0.95 = 6.082; P > 0.05) (Table 2). Results obtained from this study indicated that the seroprevalence rates of mice against *O. tsutsugamushi* were generally greater than 60% (except *S. murinus*).

**DISCUSSION**

In recent years, both reported and identified cases of scrub typhus have increased in various districts in Taiwan. Meanwhile, the identification rate of scrub typhus cases is still low (9a). Rodent mice are one of the major natural hosts of *O. tsutsugamushi*. The data of this study suggest that the increasing rate of reported cases of scrub typhus is associated with an increasing infection rate in rodent mice and that differences of mouse strains in different districts may be associated with differences in scrub typhus prevalence. Data from epidemiological surveillance and seroprevalence of antibody against *O. tsutsugamushi* among the different strains of rodent mice in different areas of Taiwan are important to programs to prevent the occurrence of scrub typhus. For reasons of safety and specificity and to meet the need for evaluation of large-scale samples in a short period of time, synthetic antigens, such as recombinant proteins, have been proposed as suitable alternatives except for antigen preparations. In this study, the recombinant truncated 56-kDa immunodominant protein from *O. tsutsugamushi* was used to develop a serological ELISA for the surveillance of IgG of rodent mice caught in districts in different geographical regions of Taiwan.

The recombinant truncated P56Δ protein, corresponding to the conserved region of the outer membrane protein among
different strains of *O. tsutsugamushi*, has been purified to near homogeneity (Fig. 1 and 2) and has been demonstrated to induce high and long-lived antibody levels in different animals, including rabbits, mice, and rats (Fig. 4 and data not shown). The difference in reactivity between the native and the recombinant protein might be due to the different interaction affinity with the rabbit anti-rP56\(\Delta\) antibody; however, the amount of native protein in the 56-kDa protein has not been measured. To resolve this problem, the infection ratio of bacteria in L929 should be calculated and cells infected with bacteria should be sorted (27). In this study, however, the purified recombinant protein was confirmed by Western blotting using mouse anti-histidine antibody (data not shown) and rabbit anti-rP56\(\Delta\) antibody.

The induced rabbit antibody was also demonstrated to recognize different strains of *O. tsutsugamushi* in both IFA and Western blotting assays (Fig. 3). Possible explanations for the different intensities of Western blot seen between various bacterial strains might be due to the different infection rates of L929 cells by various strains of *O. tsutsugamushi*. We could not rule out, however, the possibility that these differences in infection rate may have been due to different interaction affinity between various bacteria strains with rabbit anti-rP56\(\Delta\)antibody. Data obtained from L929 cells transfected with truncated 56-kDa protein expression plasmids revealed that the recognition ability of the rP56\(\Delta\)-induced rabbit antibody was almost equal for different strains of bacteria (data not shown). These findings indicate that the purified recombinant protein and its induced antibody could be used to detect antibody as well as various strains of *O. tsutsugamushi*.

A standard curve of ELISA results was established using rat antibody against rP56\(\Delta\) (data not shown), and IgG titers of rodent mouse sera were measured and adjusted by comparison to the standard curve. The recombinant ELISA used in this study was shown to have good reproducibility in comparison with IFA (\(\kappa = 0.735\); kappa statistic = 8.96; \(P < 0.05\)). Since

![FIG. 3. Antibody against recombinant outer membrane protein (rP56\(\Delta\)) can recognize different strains of *O. tsutsugamushi* by IFA. (A) IFA using rat anti-rP56\(\Delta\) antibody recognized different strains of *O. tsutsugamushi*. Mouse L929 monolayer cells infected with different strains of *O. tsutsugamushi* were assayed by IFA as described in Materials and Methods. Arrows indicate different strains of *O. tsutsugamushi* recognized by the antibody. I, II, and IV represent cells infected with the standard strains Karp, Gilliam, and Kato, respectively; III and V represent cells infected with two strains of *O. tsutsugamushi* isolated from mice captured in Kinmen County; VI represents mouse L929 cells without any infection. (B) Detection of P56 outer membrane protein of *O. tsutsugamushi* with Western blotting. Lanes: 1 to 4, L929 cells infected with Karp (lane 1), Gilliam (lane 2), Kiman (lane 3), and Kato (lane 4) strains of *O. tsutsugamushi*, respectively; lane 5, purified truncated protein (Karp strain, 31 to 274 a.a.). Arrow 1 indicates the predicted 56-kDa outer membrane protein of *O. tsutsugamushi*. Arrow 2 indicates the predicted 32-kDa truncated protein.](http://cvl.asm.org)
ELISA is easy to perform, can assay large samples in a short time, and provides semiquantitative absorbance values, it is a more suitable method for use in epidemiological surveillance of serum antibodies for rodent mice than IFA.

This study found that seroprevalence rates were significantly different among different strains of mice. Even after the exclusion of *S. murinus* from calculations, this difference remained significant ($\chi^2 = 13.62; P < 0.05$) (Table 2). Results also indicated that seroprevalence rates of mice against *O. tsutsugamushi* (except *S. murinus*) were at least 86%. In fact, scrub typhus is often underreported and may go undiagnosed (10, 21). Secondary infection is also often present subclinically in patients with scrub typhus (3). Therefore, it is very likely that many more of the people residing in the districts included in this study may be infected with *O. tsutsugamushi* or become infected in the future if proper protective measures are not instituted. Thus, our findings suggest the need for public health education in Taiwan about personal protection practices against scrub typhus and the pathogen *O. tsutsugamushi*.

Significant differences were seen in the antibody (IgG) seroprevalence rates of mice captured in different districts ($\chi^2 = 83.41; P < 0.05$). Differences in the proportion of different mouse strains might contribute to the differences in antibody seroprevalence rates of mice against *O. tsutsugamushi* among different geographic areas. For example, in this study the antibody seropositivity rates were higher in *B. indica* (100%), *R. flaviveetus* (96.17%), and *R. losea* (95.83%) than in *R. norvegicus* (86.05%) and *R. mindanensis* (83.67%) ($\chi^2 = 12.59, P < 0.05$) (Table 2). However, the major contribution to the differences in IgG seropositivity rates came from the lower seropositivity rate of *S. murinus* (54.4%). After excluding *S. murinus* from the calculation, no significant differences were found in antibody seroprevalence rates of mice from different districts ($\chi^2 = 6.082; P > 0.05$) (Table 2).

To determine whether different affinities of the secondary HRP conjugate antibody could explain the different levels of reactivity among the different species of mice, goat anti-rat or goat anti-mouse IgG-HRP secondary antibodies were used to detect rat and mouse IgG, and goat anti-*P. leucopus* IgG-HRP secondary antibody was used to detect IgG of *S. murinus*. Results showed a 77% (50 of 65) consistency between the two detection assays using different secondary antibodies (goat anti-rat IgG-HRP and goat anti-mouse IgG-HRP conjugates). This finding showed a good reproducibility ($\kappa = 0.497, z = 4.107, P < 0.05$; data not shown) between tests using these two secondary antibodies. The differences of reactivity for goat anti-rat IgG-HRP with different species of rodent mice were not significant. Thus, it seems unlikely that different levels of reactivity contributed to the different affinities of the secondary HRP conjugates. However, in the surveillance of the sero-

![FIG. 4. Recombinant protein (rP56Δ) can induce immunized animals to produce high and long-lived antibody titers. Rabbits immunized with recombinant protein induced high and long-lived titers of antibody against the outer membrane protein of *O. tsutsugamushi*. Rabbits were immunized subcutaneously twice with 40 μg of recombinant truncated protein (rP56Δ) at the 4th and 10th weeks after the basal serum collection. The sera before and after immunization were collected and mixed with 50% glycerol and stored at −20°C until use. The antibody was assayed for reactivity with rP56Δ by ELISA as described in Materials and Methods. Error bars indicate standard deviations.](http://cvi.asm.org/)

| IgG reaction obtained by ELISA | No. of IgG reactions obtained by IFA test | Total |
|-------------------------------|------------------------------------------|-------|
| +                             | 117                                      | 124   |
| −                             | 3                                        | 20    |
| Total                         | 120                                      | 144   |

* $\kappa = 0.735; z = k/\sqrt{k} = 8.96; P < 0.05.$
prevalence of antibodies, use of pooled secondary antibody mixture for different kinds of rodent mice might be helpful.

In this study, S. murinus had lower antibody (IgG) response rates, implying either that this strain of mice might not be easily infected with O. tsutsugamushi or that it could not efficiently produce antibody against the pathogen after infection. Our data obtained by nested PCR showed that S. murinus could be infected by O. tsutsugamushi (data not shown). The reasons why this strain of mice infected with O. tsutsugamushi could not induce an antibody (IgG) response efficiently remain to be determined; however, cell surface receptors or microbe-binding proteins of lymphocyte or defects of unknown components of the immune system might be involved (35). Further exploration of the mechanism of invasiveness and potential proteins that might be associated with outer membrane protein of O. tsutsugamushi is needed. Determination of the mechanism causing the inability of S. murinus to efficiently respond to the infection of O. tsutsugamushi will contribute to our understanding of host cell immunity against this pathogen and facilitate the development of methods to prevent scrub typhus.

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