Isolation and Identification of a Novel Spotted Fever Group Rickettsia, Strain IG-1, from *Ixodes granulatus*Ticks Collected on Orchid Island (Lanyu), Taiwan

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Abstract. A novel species of spotted fever group (SFG) rickettsia, *Rickettsia* spp. IG-1, was identified and isolated from adult *Ixodes granulatus* ticks collected from Orchid Island (Lanyu), an islet located in southeastern Taiwan. Serum samples collected from the rodent host *Rattus losea* and SFG-positive human serum reacted with IG-1 using an immunofluorescence assay (IFA). Pairwise nucleotide sequence analysis of 16S rRNA (rrs), gltA, ompA, ompB, and sca4 shows that IG-1 belongs to SFG rickettsiae and had the highest nucleotide sequence similarities to *Rickettsia slovaca* and *R. sibirica*. Phylogenetic analysis of the ompA, ompB genes and sca4 shows that IG-1 is most closely related to *R. honei*. The results showed the sequence divergence of this novel isolate from those rickettsiae previously characterized using the criteria proposed by Fournier and others. Whether IG-1 is pathogenic for humans remains to be studied.

INTRODUCTION

Tick-borne spotted fever group (SFG) rickettsioses are caused by obligate intracellular gram-negative bacteria belonging to the genus *Rickettsia*. At present, the genus *Rickettsia* is classified into four groups: 1) the ancestral group (AG); 2) the typhus group (TG); 3) the spotted fever group (SFG), and 4) the transitional group (TRG). 1,2,3 Although some tick-borne rickettsiae have been known to be human pathogens since the beginning of the last century, new species within the SFG rickettsiae were continuously discovered and recognized as important emerging infectious agents in the past decade. 4 All of the SFG rickettsiae are tick-borne, except *R. akari* (mite-borne) and *R. felis* (flea-borne). Humans are dead-end hosts who may show clinical symptoms of rickettsial diseases after being bitten by an infected tick. Ticks infected with pathogenic rickettsiae may transmit SFG rickettsioses, i.e., *R. rickettsii* for Rocky Mountain spotted fever (RMSF), *R. conorii* for Boutonneuse fever, *R. sibirica* for North Asian tick typhus, *R. australis* for Queensland tick typhus, *R. japonica* for Japanese spotted fever, *R. africae* for African tick bite fever, and *R. honei* for Flinders Island spotted fever.5 Most isolates of SFG rickettsiae are found in a particular geographic location and named after the place where they were first described. RMSF caused by *R. rickettsii* is reported mainly in the continental United States, except for Maine and Vermont.6 Mediterranean spotted fever (MSF) caused by *R. conorii* is endemic in the Mediterranean area, including northern Africa and southern Europe. Recently, many validated spotted fever rickettsiae species have been discovered in Asia and Australia, including *R. japonica*, *R. tamaeae*, and *R. asiatica* in Japan; *R. honei* in Thailand and Australia; *R. sibirica sibirica* and *R. sibirica mongolotimonae* in China; and *R. australis* in Australia.7,8,9 In Taiwan, tick-borne SFG rickettsioses are still poorly understood.

The microimmunofluorescence (MIF) assay remains the reference standard for serodiagnosis of SFG rickettsioses.10,11 Because of the lack of a specific reference bacterial antigen, the diagnosis often relied on a commercial MIF assay, which offered only *R. rickettsii* and *R. conorii* antigens, and positive cases were identified though serologic cross-reaction. Although it may be adequate to diagnose the SFG infection by the cross-reaction antigens, identification of the etiologic agent may be difficult. Therefore, results obtained by MIF assay should be interpreted cautiously, and specific diagnosis can only be done with cell culture isolation techniques and/or molecular techniques to identify a novel SFG *Rickettsia* species.

Development of the shell vial cell culture isolation technique and the extensive use of bacterial identification based on molecular techniques have greatly improved the detection of novel rickettsial species and the understanding of newly described rickettsioses in recent decades.12 Polymerase chain reaction (PCR) and sequencing methods are now used as sensitive and rapid tools to detect and differentiate various rickettsial species in blood and skin biopsy specimens. Primers amplifying sequences of several genes, including 16S rRNA (rrs), citrate synthase (gltA), ompA, ompB, and sca4, have been developed.13-21 According to the guidelines proposed by Fournier and others,1 to be classified as a new *Rickettsia* species, an isolate should not have exhibited more than one of the following degrees of nucleotide similarity (cut-off values), with the most homologous validated species: ≥99.8 and ≥99.9% for the rrs and gltA genes, respectively, and, when amplifiable, ≥98.8, ≥99.2, and ≥99.3% for ompA, ompB, and sca4, respectively.

Among vector-borne rickettsial diseases, scrub typhus and murine typhus are the two known zoonotic diseases in Taiwan. Scrub typhus caused by *Orientia tsutsugamushi* has been designated as a Category 4 reportable communicable disease in Taiwan since 1955 and is the most important acute febrile illness associated with the major rickettsioses. More than 16% (377 of 2,342) of the reported cases of scrub typhus were confirmed in 2006. Highly prevalent areas include Kinmen County, Penghu County, Lanyu Island of Taitung County, and Hualien County.22-25 Epidemic typhus caused by *R. prowazekii* has been designated as a Category 2 reportable communicable disease in Taiwan since 1983, although no case has ever been found in Taiwan. Instead, murine typhus (Category 4) is an endemic disease caused by *R. typhi*. Only 2.0% (25 of 1,278) of the reported cases of murine typhus were confirmed in 2006. Little is known about tick-borne SFG rickettsioses, although previous studies indicated that SFG patients may be present in Taiwan.26-28 A serologic study of...
spotted fever on field trapped rodents showed that 66.4% and 42.9% were seropositive in Kinmen County and on the main island of Taiwan, respectively. Furthermore, 27.3% of rats in Kinmen County had serum antibodies against both O. tsutsugamushi and SFG rickettsiae.25 A serosurvey of spotted fever on a normal human population in Taiwan, however, showed seroprevalence rates of 3.5–4.4%.24 More recently, three SFG Rickettsia spp., TwKM01, TwKM02, and TwKM03, were reported in Kinmen County, Lienchiang County, and Hualien County, Taiwan. Phylogenetic analysis of the partial 17-kDa antigen gene and gltA genes showed that the strain TwKM01 detected in Rhipicephalus haemaphysaloides ticks was most similar to R. rihipcephali. Two other strains, TwKM02 and TwKM03, identified in Leptotrombidium chigger mites, were most closely related to R. australis and R. felis, respectively.21 In this study, we report isolation and molecular characterization of a novel Rickettsia spp. IG-1, in Ixodes granulatus ticks collected from an aboriginal island, Orchid Island (Lanyu), Taiwan, where O. tsutsugamushi infection is endemic.

**MATERIALS AND METHODS**

**Tick collection and DNA extraction.** Ticks were collected from *Rattus lesue* captured by setting up live traps in the fields of Orchid Island (Lanyu), Taiwan, in September 2006. All ticks collected from each rodent were kept humid in plastic vials and preserved in the refrigerator at a temperature of 4°C. Ticks were identified by standard taxonomic characteristics and 16S rDNA analysis.26,27 Each tick was surface disinfected by immersion in iodinated alcohol for 10 minutes, rinsed with sterile distilled water twice, and homogenized and triturated in 0.3 mL of Eagle minimum essential medium (MEM) containing 4% fetal bovine serum for later DNA extraction and rickettsia isolation. DNA was extracted from each tick sample and/or infected L929 cells with the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.

**Cell culture and rickettsia isolation.** L929 mouse fibroblast cells (ATCC CCL 1 NCTC Clone 929) were cultured in MEM medium containing 4% fetal bovine serum (Gibco-BRL, Grand Island, NY) in T-75 flasks. Briefly, culture was performed using the centrifugation shell vial technique (Sterilin-Felthan-England, 3.7 mL) containing 12-mm-round coverslips seeded with 5 × 10^4 L929 cells incubated in a 5% CO2 incubator at 37°C for 2 days to obtain a confluent monolayer.28 The vial containing the tick inoculum was centrifuged at 700 × g for 1 hour at 22°C. After decantation of the inoculum, two washes with sterile phosphate-buffered saline (PBS) were performed, and fresh MEM containing 4% fetal bovine serum and 2 mol/L L-glutamine medium was added and incubated at 32°C under a 5% CO2 atmosphere.29 The cultures were replaced with fresh medium every week. Rickettsiae were propagated in three shell vials with L929 cells and detected by PCR targeting the rickettsia gltA gene, Gimenez staining, and/or immunofluorescence assay (IFA) every week.28,30 Positive shell vials were harvested and inoculated into a T-25 flask containing a monolayer of confluent L929 cells.

**Fluorescence microscopy.** The infected L929 cells on chamber slides were fixed and permeabilized with ice-cold acetone/methanol (1:1) at −20°C for 10 minutes. The slides were dried and blocked with PBS containing 1% goat serum. Either positive control human serum against spotted fever (1:100 dilution; PanBio, Columbia, MD) or rodent host *Rattus lesue* serum (1:50 dilution) was used as the primary antibody. Bacteria were subsequently labeled with a fluorescein conjugated goat anti-human or mouse immunoglobulin G (IgG; Sigma, St. Louis, MO) diluted 1:200 in PBS containing 2.0% Evans blue. After staining of bacteria, slides were washed three times with PBS containing 0.1% Tween 20 and mounted to glass coverslips using mounting medium (PBS:glycerol = 3V:7V) and examined at ×400 with a Zeiss fluorescent microscope.

**PCR amplification and DNA sequencing.** PCR amplification was accomplished in 50-μL volumes according to the manufacturer’s suggested protocol (QIAGEN). The reaction mixture contained 0.2 μmol/L each of the rickettsia-specific primer sets, 10 mmol/L of dNTP mixture, 10× QIAGEN PCR buffer, 5× Q-Solution, and 2.5 U of Taq DNA polymerase (QIAGEN). Nested PCR was used in single tick detection for the Rickettsia gltA gene (outer primer pair: RpCS.877p: 5′-GGG GCC CTG CTC ACG GCC G-3′; RpCS.1258n: 5′-ATT GCA AAA AGT ACA GTG A-3′). inner primer pair: RpCS.896p: 5′-GCC TAA TGA AGC AGT GAT AA-3′; RpCS.1233n: 5′-GCC AGC GTA TAC CCA TAG C-3′) and ompB gene (outer primer pair: rompB OR: 5′-GCT GGA AGT AAT CGT TTC GTA A-3′; rompB ON: 5′-GCT TTA TAA CCA GCT AAA CC CC-3′). inner primer pair: rompB SPG IF: 5′-GTT TAC TAC GTG CTG CTA ACC AA-3′; rompB SPG/IG TR: 5′-GTT TGG GCC CAT ATA CCA TAA G-3′). After electrophoresis, gels were stained with ethidium bromide (EtBr), and amplicons were visualized with a UV transilluminator. PCR products were purified using the QIAGen Gel Extraction Kit (QIAGEN). PCR amplification and sequencing of the rrss, gltA, ompA, ompB, and sca4 were performed using the primers and conditions described previously.17–21 PCR products were sequenced twice in each direction. Molecular identification of the tick from the 3′ end of the mitochondrial 16S rDNA gene (rDNA) was performed according to a previous report.26

**Analysis of sequences and construction of phylogenetic trees.** Sequence analysis was performed with the Lasergene software packages (DNASTAR, Madison, WI), and multisquence alignment was made with MEGA 4.32 Calculation of sequence similarity has been described previously.1 Phylogenetic trees were obtained from DNA sequences by using the neighbor-joining and maximum-parsimony methods (CLUSTAL software in MEGA 4 and PHYLIP version 3.6).33 Genetic distances were calculated using the Kimura two-parameter distance algorithm. The phylogenetic tree from the concatenated datasets of four genes sequences (gltA, 16S rRNA, sca4, and ompB) was constructed as previously reported.24 Briefly, multisquence alignment of each gene was made with Clustal X software package by MEGA 4. The alignments were concatenated to produce a single multiple alignment of 10,481 nucleotide sites. One thousand bootstrap replicates were performed to estimate the node reliability of the phylogenetic trees obtained by the two methods. Bootstrap support values > 75 are considered significant.

**Nucleotide sequence accession number.** The GenBank nucleotide sequence accession numbers for the nucleotide sequence data generated in this study are EF896608, EF219460, EF219461, and EF219462 for partial sequences of *rrss*, *gltA*, *ompA* (apart from the tandem repeat units), *ompB*, and *sca4* of *Rickettsia* spp. IG-1, respectively. The GenBank
nucleotide sequence accession number for partial 16S rDNA gene sequence of *Ixodes granulatus* tick is EF427898. Accession numbers of the gene sequences used in this study were indicated within the figures.

**RESULTS**

**Detection of rickettsiae in ticks.** Four adult *I. granulatus* female ticks collected from four individual host *R. losea* were examined in this study. DNA extracted from these ticks was tested by PCR using primers targeting the *gltA* and *ompB* genes of *Rickettsia* species. All of four samples yielded the expected nested-PCR product of 426 and 338 bp, respectively. *Rickettsia* species were successfully isolated in L929 cell culture from all four PCR-positive ticks using the shell vial centrifugation technique. Analysis of DNA sequences of the *gltA* and *ompB* genes showed that these isolates were 100% identical to each other.

**Gimenez staining and IFA on Rickettsia spp. IG-1 isolate.** The *Rickettsia* spp. IG-1 in L929 cell culture was shown by Gimenez staining (data not shown) and IFA (Figure 1). Serum collected from the rodent host *R. losea* showed positive staining when tested with the antigen of *Rickettsia* spp. IG-1 (Figure 1A). Furthermore, immunostaining with commercial positive SFG human serum (Figure 1B) showed rod spots in the cytoplasm of L929 cells.

**Molecular characterization of the new isolate.** Comparison of nucleotide sequences obtained from the IG-1 isolate with those sequences available in GenBank showed that *R. slovaca* is the closest SFG rickettsiae to *Rickettsia* spp. IG-1, with nucleotide similarity of 99.6%, 99.1%, 98.1%, 98.6%, 98.3%, and 98.7% for *rrs*, *gltA*, the 5' end of *ompA*, the 3' end of *ompA*, *ompB*, and *sca4*, respectively. Table 1 summarizes the corresponding closest relative sequences to each of the gene fragments. These results also validate the divergence of this novel isolate from those rickettsiae previously characterized using the criteria proposed by Fournier and others. Partial sequences of the *gltA* and *ompB* genes from both tick inocula

**FIGURE 1.** L929 cells infected by *Rickettsia* spp. IG-1 as shown by immunofluorescence assay using rodent host serum and anti-mouse IgG-fluorescein conjugates (A) and human anti-*Rickettsia* serum and anti-human IgG-fluorescein conjugates (B). This figure appears in color at www.ajtmh.org.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence length (bp)</th>
<th>Length of ORF to <em>R. rickettsii</em></th>
<th>Results (% pairwise nucleotide sequence similarity* with phylogenetically closest <em>Rickettsia</em> species from NCBI)</th>
<th>Cut-off values of Fournier and others</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrs</em></td>
<td>1,381</td>
<td>44–1,424</td>
<td>99.6% to <em>R. slovaca</em> (L36224); <em>R. raoultii</em> (DQ365809); <em>R. conorii</em> strain Malish 7 (AE086647); <em>R. peucostickii</em> (DQ024333); <em>R. rickettsii</em> strain R (L36217); <em>Rickettsia</em> spp. R (U25042); <em>R. sibirica</em> (L36218); <em>R. sibirica mongolotimonae</em> (L36219); <em>R. massiliae</em> (L36106); <em>R. honei</em> TT118 (L36220)</td>
<td>99.8%</td>
</tr>
<tr>
<td><em>gltA</em></td>
<td>1,051</td>
<td>87–1,134</td>
<td>99.2% to <em>R. sibirica</em> (U59734); <em>R. BJ-90</em> (AF178035); 99.1% to <em>R. slovaca</em> (U59725); <em>R. honei</em> TT-118 (U59726); <em>Rickettsia</em> spp. S (U59735); <em>R. conorii</em> subsp. caspia (U59728); <em>R. parkeri</em> (U59732)</td>
<td>99.9%</td>
</tr>
<tr>
<td>5’ end of <em>ompA</em>†</td>
<td>589</td>
<td>1–590</td>
<td>98.1% to <em>R. slovaca</em> (U43808)</td>
<td>98.8%</td>
</tr>
<tr>
<td>3’ end of <em>ompA</em>‡</td>
<td>3,182</td>
<td>3,071–6,252</td>
<td>98.6% to <em>R. slovaca</em> (U83454); <em>R. honei</em> TT-118 (U83456); <em>R. honei</em> strain RB (AF018075)</td>
<td>99.3%</td>
</tr>
<tr>
<td><em>ompB</em></td>
<td>4,840</td>
<td>296–5,141</td>
<td>98.2% to <em>R. conorii</em> (U83437); 98.3% to <em>R. slovaca</em> (AF123732); 97.8% to <em>R. africani</em> (AF123706); <em>R. honei</em> strain RB (AF123711); <em>R. parkeri</em> (AF123717); <em>R. sibirica</em> (AF123722)</td>
<td>99.2%</td>
</tr>
<tr>
<td><em>sca4</em></td>
<td>2,947</td>
<td>33–2,979</td>
<td>98.7% to <em>R. slovaca</em> (AF155054); 98.5% to <em>R. honei</em> subsp. marmonii (DQ300905); <em>R. africani</em> (AF151724); <em>R. conorii</em> subsp. caspia (AF163007); <em>R. honei</em> TT-118 (AF163004)</td>
<td>99.3%</td>
</tr>
</tbody>
</table>

* Gap and missing data were estimated by pairwise deletion, similarity = 1 – P-distance.
† *ompA* fragment I.
‡ *ompA* fragments II, III, IV, and V.
§ Length of ORF to *R. africani* (U83436).
(before) and isolation culture (after) of four isolates showed that they are completely identical to each other.

Phylogenetic analysis from concatenated datasets of four genes sequences (gltA, 16S rRNA, sca4, and ompB) found that Rickettsia spp. IG-1 formed a separate clade from the other rickettsiae in both neighbor-joining (Figure 2A) and maximum parsimony (data not shown) methods. This pattern confirms the existence of the novel species of the genus Rick-
ettia in Taiwan. Phylogenetic trees of the ompA (Figure 2B) and ompB genes and sca4, which encoded major outer-membrane proteins, constructed using neighbor-joining and maximum parsimony (data not shown) methods showed that isolate IG-1 is most closely related to R. honei. The bootstrap values at the node for IG-1 sequences and its closest relatives are 35.6, 36.1, 65.4, 72.3, 80.2, and 81.4 for gltA, 16S rRNA (rs), ompA, ompB, sca4, and concatenated sequences (gltA-16S rRNA-sca4-ompB) using PHYLIP versus 24, 35, 52, 66, 86, and 78 using MEGA 4, respectively.

DISCUSSION

In this study, we report the first isolation and molecular characterization of a novel spotted fever group (SFG) rickettsia, Rickettsia spp. IG-1, isolated from I. granulatus ticks from an aboriginal island, Orchid Island (Lanyu), Taiwan. Using gene sequence-based molecular identification methods, five genes (rs, gltA, ompA, ompB, and sca4) have been proposed to identify new Rickettsia isolates.1 The novel spotted fever group (SFG) rickettsia, Rickettsia spp. IG-1, was found to have high similarity to R. slovaca and R. honei. R. slovaca, transmitted by Dermacentor marginatus and D. reticulatus, has been identified mainly in Europe as the causal agent of tick-borne lymphadenopathy (TIBOLA).35 The SFG rickettsia with the widest recognized distribution is R. honei. This rickettsia had exceptionally wide distribution in the areas of Southeast Asia (Thailand), Australia (Flinders Island), and North America (south Texas) and has been detected in Aponomma hydroasuri, Amblyomma cajennense, I. granulatus, and Haemaphysalis novaeguineae ticks.8,9,36–38 With the growing list of newly discovered rickettsiae in recent years, such as R. tamaruae and R. asiatica in Japan, it is expected that more new SFG rickettsiae species will be discovered in Asia.16

The geographic origin of the human infection has been one of the best indicators of Rickettsia species identity. Testing a clinical serum against the possible Rickettsia species known to occur in a given area will provide the optimal assay condition because homologous antibody titers induced are often higher than heterologous antibody titers.15 Although the diversity of the genus Rickettsia spp. had been shown in spotted fever, rodent host serum and human SFG antiserum could cross-react with a genus-specific antigen. These cross-reacting antibodies were also observed between biogroups (SFG and typhus group) against lipopolysaccharide that mainly induced IgG antibodies were also observed between biogroups (SFG and typhus group) against lipopolysaccharide that mainly induced IgM antibody.39,40 Most SFG rickettsiae isolates found in a particular geographic location have relatively minor antigenic and genomic differences, suggesting local evolution from a relatively recent common source proto-SFG-rickettsia. It is speculated that serologic tests using specific spotted fever group rickettsiae, such as Rickettsia spp. IG-1, would improve the accuracy of serodiagnosis of rickettsioses in Taiwan. We reason that more tick species belonging to the genera Ixodes, Haemaphysalis, Dermacentor, Amblyomma, Rhipicephalus, Boophilus, Argas, and Ornithodoros are worth further study. Considering the large number of tick species present in Taiwan, and their infection with different spotted fever group rickettsiae, identifying the tick species responsible for a bite could be helpful for accurate diagnosis.

In conclusion, our report describes the first isolation and molecular characterization of a novel SFG Rickettsia, a tentatively named Rickettsia spp. IG-1. On the basis of phylogenetic analysis, we believe that IG-1 is genetically unique and propose a novel SFG rickettsia named Candidatus Rickettsia lanyuenensis, after the Lanyu Island. This report also highlights the potential threat of tick-borne rickettsial disease in Taiwan. Whether I. granulatus ticks can serve as vectors of rickettsiae to humans or other small mammals in maintaining and spreading rickettsiae in nature remains to be studied.

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