Molecular Detection and Characterization of Spotted Fever Group Rickettsiae in Taiwan

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Abstract. Rickettsioses are emerging infectious diseases caused by rickettsiae in association with arthropods. We report the detection of spotted fever group rickettsiae (SFGR) in Taiwan using molecular methods. Phylogenetic analyses of the 17-kd protein and citrate synthase (gltA) genes showed that SFGR TwKM01 detected in Rhipicephalus haemaphysaloides ticks was most similar to Rickettsia rhipicephali. Three TwKM01 isolates were obtained from three individual R. haemaphysaloides ticks. Small, intracellular, coccobacillary bacteria were found in infected L929 cells using immunofluorescence antibody testing and transmission electron microscopy. Two other SFGRs, TwKM02 and TwKM03, identified in Leptotrombidium chigger mites, were closely related to R. australis and R. felis URRWXCal2, respectively. The TwKM03 strain was also detected in Ixodes granulatus ticks and widely distributed in Hualien, Kinmen, and Lienchiang counties in Taiwan. The endonucleases MaeII and Hhal selected for restriction fragment length polymorphism analysis of the gltA and 17-kd polymerase chain reaction products, respectively, were useful for genotyping Rickettsia species TwKM01, TwKM02, TwKM03, and other SFGRs. Although their infectivity and pathogenicity for vertebrates are unknown, the finding of SFGRs raises the possibility that bacteria other than Orientia tsutsugamushi, Coxiella burnetii, and R. typhi may be involved in rickettsial diseases in Taiwan.

INTRODUCTION

Rickettsiae are a group of obligate intracellular gram-negative bacteria that cause many emerging infectious diseases throughout the world.1 The rickettsioses often share characteristic clinical features including fever, rash, headache, and sometimes, eschar. In Taiwan, scrub typhus is an endemic rickettsial disease reported mainly in eastern Taiwan and its offshore islands, but sporadic cases are also reported in almost every county.2 In recent years, the annual number of confirmed cases of scrub typhus in Taiwan increased from 23 cases in 1991 to 462 cases in 2005. However, many suspected cases reported to the Centers for Disease Control (CDC) in Taiwan remain unconfirmed (e.g., 1,590 cases in 2004; data from the Epidemiology Bulletin, CDC, Taiwan). Two other rickettsial diseases, murine typhus and Q fever, have also been identified by serologic methods recently.3−5 However, detailed data on the epidemiology and pathogenesis of both diseases in Taiwan are still lacking. Clinical human cases of rickettsial diseases other than scrub typhus, Q fever, and murine typhus have not yet been reported, especially diseases of the spotted fever group (SFG) of rickettsioses.

The number of newly described SFG rickettsiae (SFGRs) has increased in recent decades.6 After the discovery of Japanese spotted fever, caused by Rickettsia japonica, in 1984, six other typical Japanese SFG isolates were also isolated from six different tick species by polymerase chain reaction (PCR) and monoclonal antibody testing.7−9 Recently, Choi and others10 also studied SFGRs in patient sera that were seropositive for SFGRs, suggesting that several kinds of rickettsial diseases are occurring in Korea. In China, 30 SFGRs were isolated from human patients, vector ticks, and many rodent hosts in different geographic areas including Heilongjiang, Xinjiang, Inner Mongolia, Hainan, Fujian Provinces, and Beijing City.11

Both Kinmen and Lienchiang counties of Taiwan are geographically located closer to Fujian province of China than to Taiwan (Figure 1). Many identified tick and rodent hosts, such as Ixodes granulatus (tick), Rhipicephalus haemaphysaloides (tick), and Rattus flavipectus (rat), common to Mainland China are also commonly seen in Kinmen and Lienchiang counties and in Taiwan as well. Many zoonotic diseases may, therefore, be transmitted from Mainland China to Taiwan through the smuggling of live animals from the two islands. Although a human serosurvey of spotted fever showed that rickettsioses were not very prevalent (3.5−4.4%) in Taiwan, another earlier study by Chen and others2,3 reported surprisingly contradictory results: 66.4% and 42.9% of field trapped rodents were seropositive for spotted fever in Kinmen county and on Taiwan, respectively. This indicated that SFGRs could be present in Taiwan but not yet discovered.12

Although various Rickettsia species are found in ticks and some arthropods from Japan and China, to date, no studies have been conducted on potential rickettsiae in Taiwan arthropods.13−15 In this study, to show whether or not SFGRs were present in Taiwan, ticks and other ectoparasitic specimens on each field-trapped rodent from Kinmen and Lienchiang counties and Taiwan were studied. This study presents the first molecular evidence and characterization of three SFGR strains in arthropods and rodents in Taiwan.

MATERIALS AND METHODS

Arthropod collection and DNA extraction. All ectoparasitic arthropods were collected from rodents captured by setting live traps in the fields of Kinmen county (in May 2002), Lienchiang county (in July 2002), and Hualien county (twice in January 2001 and April 2004) of Taiwan. Ticks, Leptotrombidial chigger mites, and Mesostigmatal mites collected from each rodent were identified and classified, and same-species

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samples were pooled together as one sample. The arthropod specimens were surface disinfected by 5-minute immersion in iodinated alcohol, rinsed with sterile diethylpyrocarbonate-treated water, and triturated in 0.2–1.0 mL sucrose phosphate glutamate (SPG) buffer for later extraction of total DNA and rickettsial cultivation in cell cultures. DNA was extracted from each arthropod sample and/or infected cell using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany).

**PCR amplification and restriction length polymorphism (RFLP).** PCR amplification was accomplished in 50-μL volumes as described previously.

Nested PCR was used for gltA gene amplification (primer pair: Rp877p and Rpl258n), whereas PCR was used for the 17-kd protein gene (primer pair: Rr17.13, TAGAGAATTATGGAAC-TATTATC; Rr17.495r, ATGACGTGTTTGTCTATCA-TCAC). PC reactions were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Reno, NV).

RFLP analyses were used for the characterization of rickettsia-specific 17-kd protein and gltA gene PCR products. Diagnostic restriction enzymes were selected by the aid of restriction mapping software Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/). Both the 17-kd antigen and the gltA gene PCR products were digested with HhaI and MaeII (New England BioLabs, Boston, MA), respectively. The digestion products were separated through electrophoresis on a 2% agarose gel, stained with ethidium bromide (EtBr), visualized by UV illuminator, and photographed with Polaroid type 655 P/N film (Polaroid, Cambridge, MA).

**DNA sequencing and phylogenetic analysis.** After purification using the QIAquick PCR purification kit (Qiagen), 17-kd protein and gltA gene PCR products corresponding to the Rr17.13-Rr17.495r and CS882-CS1261r primer pairs were sequenced twice in the forward and reverse directions. The sequences from representative rickettsial species chosen to infer the phylogenetic trees of gltA and 17-kd genes were multialigned using Clustal X software. Phylogenetic trees were constructed by the neighbor-joining method using MEGA version 3.1 software (http://www.megasoftware.net/index.html).

**Nucleotide sequence accession numbers.** Except for the 17-kd rickettsial protein from strain TwKM02, the nucleotide sequences of the gltA gene and 17-kd protein PCR products from the three rickettsial strains studied, TwKM01, TwKM02, and TwKM03, were deposited in the GenBank database under accession numbers AY445819, AY445820, AF540555, AY445821, and AY445822, respectively. The accession numbers of these two genes from other representative rickettsial species are shown in Figures 2 and 3. The accession numbers of the partial ompB gene sequence of rickettsial species TwKM02 is EF364045.

**Isolation of rickettsiae from arthropods.** Briefly, each pooled tick or mite homogenate in SPG buffer as described
above was centrifuged at 250g for 3 minutes at 4°C to collect the supernatant and remove the debris. The supernatant was centrifuged at 10,000g for 5 minutes to pellet the rickettsiae. The pellet was washed twice with phosphate-buffered saline (PBS) and resuspended in modified Eagle’s minimum essential medium (MEM) containing 4% fetal bovine serum (FBS) to inoculate confluent monolayers of L929 cells in a 12-well tissue culture plate. The plate containing the inoculate was centrifuged at 700g at 25°C for 1 hour. After decanting the supernatant, new MEM medium was added, and the plate was incubated at 34°C for 7–14 days in a CO₂ incubator. Assessment of the inoculated cell cultures for presence of infection was performed using PCR and immunofluorescence antibody (IFA) testing.

**IFA test.** Rickettsial infection was determined using an IFA test for detection of rickettsial antigen. Briefly, the infected L929 cells on chamber slides were fixed and permeabilized in cold acetone at −20°C for 10 minutes. The slides were dried and blocked with PBS containing 1% goat serum. The primary antibodies used in indirect immunofluorescence labeling of intracellular bacteria were standard SFG-positive human serum (PanBio, Brisbane, Australia). Bacteria were subsequently labeled with goat anti-human IgG antibodies conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich, St Louis, MO). Whenever necessary, cells were also treated with 0.003% Evans blue for background staining. After staining of bacteria, slides were washed three times in PBS and mounted to glass coverslips using mounting medium (DAKO, Carpinteria, CA) and examined under an epifluorescent microscope (Olympus, Tokyo, Japan).

**Transmission electron microscopy.** Transmission electron microscopy (TEM) was conducted on L929 cells infected with rickettsiae after 14 days of incubation. Ultrastructural studies were done according to previously described methods. In brief, the infected L929 cell pellets were fixed in cold 2% glutaraldehyde for 2 hours and postfixed with freshly prepared 1% osmium tetroxide for 2 hours. The specimens were dehydrated in a graded series of ethanol solutions and embedded in Spurr resin and polymerized at 70°C. Thin sections were cut on an ultra-thin section microtome and doubly stained with uranyl acetate and lead citrate before observation with the electron microscope (75 kV; H-600; Hitachi, Tokyo, Japan).

**RESULTS**

**Host and arthropod collection.** A total of 280 rodents were trapped for ectoparasitic arthropod collection from Kinmen (in May 2002), Lienchiang (in July 2002), and Hualien counties in Taiwan (in January 2001 and April 2004). Two rodent species (Rattus flavipectus and R. norvegicus) and one insectivora species (Suncus murinus, Asian musk shrew) were trapped in Kinmen county, two rat (R. losea and R. norvegicus) and one insectivora species (S. murinus) in Lienchiang county, and another five rodent species (R. losea, R. norvegicus, R. exulans, Bandicota indica, and Apodemus agrarius) and one insectivora species (S. murinus) were trapped in Hualien county. A total of 88 tick pools, 112 Leptotrombidial chigger mite pools, and 85 Mesostigmata mite pools were collected from the trapped rodents. The *Rhipicephalus haemaphysaloides* tick, *Ixodes granulatus* tick, *Leptotrombidium deliense* chigger mite, and Mesostigmata mite were the predominant species of ectoparasites among those animals.

**PCR and sequence analysis.** A partial *gltA* sequence was detected in six tick pools (*R. haemaphysaloides*) and four chigger mite pools (*L. deliense*) collected in Kinmen county, one tick pool (*I. granulatus*) and one chigger mite pool (*L. deliense*) in Lienchiang county, and two tick pools (*R. haemaphysaloides*) in Hualien county (Figure 1; Table 1). We also

![Figure 3](image-url)  
**Figure 3.** RFLP analyses of rickettsial citrate synthase (*gltA*) and 17-kd antigen genes. Lanes M1 and M2: 1-kb Plus DNA ladder and pBR322 DNA-MspI digest. Lanes 1 and 8: PCR-negative controls of citrate synthase (*gltA*) or 17-kd antigen genes. Lanes 2, 4, and 6: uncut 380-bp *gltA* PCR products of *Rickettsia* sp. TwKM01, TwKM02, and TwKM03. Lanes 3, 5, and 7: *MaeII*-digested PCR products of TwKM01, TwKM02, and TwKM03. Lanes 9 and 11: uncut 508-bp 17-kd antigen PCR products of TwKM01 and TwKM03. Lanes 10 and 12: *HhaI*-digested PCR products of *R*. sp. TwKM01 and TwKM03.

<table>
<thead>
<tr>
<th>Strains of SFG</th>
<th>SFG cluster</th>
<th>Identity (%)</th>
<th>Arthropod</th>
<th>Rodent</th>
<th>Location</th>
<th>Pool no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rickettsia sp. TwKM01</td>
<td><em>R. rhipicephali</em></td>
<td>99.2</td>
<td>99.4</td>
<td>Rh</td>
<td>Rf</td>
<td>KM</td>
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<tr>
<td>Rickettsia sp. TwKM02</td>
<td><em>R. australis</em></td>
<td>98.4</td>
<td>NA</td>
<td>Ld</td>
<td>Rf</td>
<td>KM</td>
</tr>
<tr>
<td>Rickettsia sp. TwKM03</td>
<td><em>R. felis</em></td>
<td>99.2</td>
<td>100.0</td>
<td>Ld</td>
<td>Sm</td>
<td>LC</td>
</tr>
</tbody>
</table>

* SFGR with highly similar sequences present in GenBank.
found one gltA PCR-positive in one Mesostigmata mite pool from Hualien county. To our knowledge, this is the first report of *Rickettsia* spp. other than *Orientia tsutsugamushi* detected in *L. deliense* chigger mites. The prevalence of rickettsia infection in tick and mite pools was 5.9% (2/34) and 1.2% (1/85) in Hualien county, 14.3% (6/42) and 7.5% (4/53) in Kinmen county, and 8.3% (1/12) and 10% (1/10) in Lienchiang county, respectively.

All 15 gltA positive samples were sequenced and compared with other SFG and typhus group (TG) rickettsiae to determine pairwise similarities using the DNASTar (DNASTAR, Madison, WI) and ClustalX multiple alignment programs. Eight sequences from *R. haemaphysaloides* were identical to one another and were named *Rickettsia* species TwKM01, with 99.2% identity to *R. rhipicephali*. Three samples from *L. deliense* mite pools of Kinmen county and Lienchiang county were identical and were named *Rickettsia* species TwKM02, with 98.4% similarity to *R. australis*. Two sequences detected in *L. deliense* chigger mite pools from Kinmen county, one sequence in the *I. granulatus* tick from Lienchiang county, and one sequence in the Mesostigmata mite pool from Hualien county were all identical and were most similar to *R. felis* URRWXCaI (99.2%) and were called *Rickettsia* species TwKM03.

The 17-kd protein nucleotide sequences were also amplified to confirm the cluster patterning of gltA sequencing (Table 1). The 17-kd partial sequences of samples from the same strains, TwKM01 or TwKM03, were all identical and were most similar to *R. rhipicephali* (99.4%) and *R. felis* URRWXCaI (100%), respectively. However, the expected 508-bp PCR product of TwKM02 strain was not detected in the *L. deliense* chigger mite pools from either Kinmen or Lienchiang county because of PCR failure.

Although the 17-kd protein nucleotide sequence failed to be amplified in the *L. deliense* chigger mite pools from either Kinmen or Lienchiang counties, an 812-bp partial ompB gene product was amplified instead. The ompB partial sequences (accession number EF364045) of samples in the same strain TwKM02 were all identical and were most similar to *R. australis* (95% identical). The result was in agreement with the result for gltA gene (Table 1).

**Phylogenetic analysis.** Phylogenetic trees based on the partial gltA and 17-kd protein gene sequences were inferred using the neighbor-joining method to study the relationships among the rickettsial species. As shown in Figure 2, *Rickettsia* species TwKM01 was most closely related to *R. rhipicephali* and fell into the *R. rhipicephali* cluster together with the R300 strain, Bar 29 strain, and *R. aeschlimannii*. On the other hand, *Rickettsia* species TwKM02 and TwKM03 belonged to the *R. australis* and *R. felis* URRWXCaI clusters, respectively. The dendrogram inferred from the 17-kd protein sequences (Figure 3) was similar to the one from gltA sequencing (Figure 2) using the neighbor-joining method, except that the 17-kd protein nucleotide sequence of *Rickettsia* species TwKM02 was not available because of PCR failure (Figure 3).

**PCR-RFLP analysis.** Using Webcutter 2.0 software, the endonucleases *MaeII* and *HhaI* were selected for RFLP analysis of the gltA and 17-kd protein PCR products, respectively, to differentiate the species of rickettsia. The estimated restriction map of these gltA products with *MaeII* (Figure 4) revealed that *Rickettsia* species TwKM01, TwKM02, and TwKM03 had the same migration patterns as *R. rhipicephali*, *R. australis*, and *R. felis* URRWXCaI, respectively, and differed from other clusters of rickettsiae. As shown in Figure 3, the digestion patterns of *Rickettsia* species TwKM01, TwKM02, and TwKM03 among the validated SFGRs. *R. bellii* was used as the outgroup. Bootstrap values > 50% are indicated at the nodes of the phylogenetic tree. The scale bar indicates a 1% nucleotide sequence divergence.

![Figure 4. Neighbor-joining phylogram based on partial gltA sequences showing the phylogenetic placement of *Rickettsia* sp. TwKM01, TwKM02, and TwKM03 among the validated SFGRs. *R. bellii* was used as the outgroup. Bootstrap values > 50% are indicated at the nodes of the phylogenetic tree. The scale bar indicates a 1% nucleotide sequence divergence.](image-url)

**Isolation of rickettsiae.** To evaluate the isolation of rickettsiae from ticks, the infected L929 cell cultures were monitored using PCR and IFA staining. Although no cytopathic effect was observed in any infected cell culture, three *Rickettsia* sp. TwKM01 isolates, TwHL03, TwHL04, and TwHL05 were isolated from *R. haemaphysaloides* ticks from three individual *Bandicota indica* in Hualien county, Taiwan. The PCR sequences of gltA and 17-kd protein genes of TwHL03, TwHL04, and TwHL05 isolates were completely identical to the TwKM01 strain and are thought to be the same strain.

Small, intracellular, coccobacillary bacteria were revealed in infected L929 cells using IFA staining (Figure 6). The isolates grew slowly in L929 cells, and often a few scattered cells...
Rickettsia were superinfected in the same chigger mite or were because humans are accidentally bitten by these species.

TwKM01 was used as the outgroup. Bootstrap values > 50% are indicated at the nodes of the phylogenetic tree. The scale bar indicates 2% nucleotide sequence divergence.

showed the rickettsial infection even after 10 days of culture. The optimum conditions for Rickettsia TwKM01 cultivation remain under study.

Ultrastructure of Rickettsia sp. TwKM01. The morphologic fine structures of the three Rickettsia species TwKM01 isolates were also very similar to one another on electron microscopy. The rickettsiae were present and free in the cytoplasm of the L929 cells (Figure 7A). The rickettsiae measured ~1–1.5 μm in length and 0.5 μm in diameter (Figure 7, A and B). Binary fission of the rickettsiae was observed (Figure 7A). The trilaminar cell wall consisted of an inner leaflet, an outer leaflet, and clear space between the inner and outer leaflets. An electron lucent halo zone, measuring ~30 nm, was seen around the cell wall (Figure 7B).

DISCUSSION

This study represents the first molecular detection and characterization of SFGRs in Taiwan. The three SFGR species TwKM01, TwKM02, and TwKM03 are closely related to Rickettsia rhizophelphi, R. australis, and R. felis URRWXCal2. Whether they are pathogenic to humans in Taiwan is unknown; however other rickettsiae, such as R. aeschlimannii, R. felis, R. helvetica, R. marmorii, R. massiliae, R. parkeri, R. sibirica strain mongolotimonae, and R. slovaca, of previously unknown pathogenicity, were subsequently associated with human diseases.31–30

Rickettsia species TwKM01 was identified in R. haemaphysaloides ticks (Table 1). Although the R. haemaphysaloides tick is also found in Fujian and other southern provinces of China, it was the first reported tick to carry SFGRs in Taiwan.31 Because humans are accidentally bitten by these three-host ticks, R. haemaphysaloides may transmit SFGRs to humans from other wild natural reservoirs.31 In addition, the R. haemaphysaloides tick is a common ixodid species in China, India, and other south Asian countries and is a major vector of bovine babesiosis in China and SFGR RH-1 in Nepal.32–33 Rickettsia species TwKM02 was first and only detected in L. deliense chiggers in Taiwan and is different from R. australis detected in Ixodes sp. ticks in Australia and R. akari (97.3% identity for gltA) detected in Liponyssoides sanguineus sibiriaca in New York.16 Recently, a similar molecular evidence shows the predominant Rickettsia species that was found in chigger mites is closely related to Rickettsia species TwKM02 in Korea.34 To our knowledge, most SFGRs are transmitted by ixodid ticks, except for R. akari that causes rickettsialpox and is transmitted by the bite of the house mouse mite L. sanguineus.35 Interestingly, Rickettsia species TwKM03, which has many arthropod hosts in Taiwan, may be classified to form a cluster with R. felis URRWXCal2 and Rickettsia sp. California 2 in the United States, both having been detected in fleas.17 According to the gene sequence based criteria outlined by Fournier and others,9 Rickettsia species TwKM01 or TwKM02 could be novel species by citrate synthase gene analyses and need to be further confirmed by the gene analyses for ompA, ompB, and Gene D.

Interestingly, some TwKM02 strains were revealed to coinfect with O. tsutsugamushi in the same chigger mite pools, using PCR analysis of the 56-kd protein gene of O. tsutsugamushi (data not shown). Whether the Rickettsia species TwKM02 was obtained from the blood meal of an infected rodent or from the mite tissue needs further study. It was not known whether Rickettsia species TwKM02 and O. tsutsugamushi were superinfected in the same chigger mite or were infected in different chiggers but in the same chigger pool. In either condition, the two pathogens co-existed in the same chigger mite population, which could facilitate their co-

**Figure 5.** Neighbor-joining phylogram based on partial 17-kd protein gene sequences showing the phylogenetic placement of Rickettsia sp. TwKM01, TwKM02, and TwKM03 among the validated SFGR. R. bellii was used as the outgroup. Bootstrap values > 50% are indicated at the nodes of the phylogenetic tree. The scale bar indicates 2% nucleotide sequence divergence.

**Figure 6.** Immunofluorescence staining of Rickettsia sp. TwKM01 in infected L929 cells. Cells were treated with 0.01% Evans blue for background staining. A, L929 cells only. B, Rickettsia sp. TwKM01-infected L929 cells. This figure appears in color at www.ajtmh.org.
infection in a mammalian host when the chigger mites bite. The high prevalence of antibodies against both *O. tsutsugamushi* and SFGR had been detected among rats (27.3%) in Taiwan and human (31.1%) in Malaysia, suggesting the possibility of co-infection, previous exposures, or serological cross-reactions. It will be interesting to study whether the co-infected chigger mites can transmit both diseases at the same time and what symptoms will be shown. Moreover, because there has been no SFGR-infected human case reported in Taiwan before, and the febrile symptom of spotted fever is also similar to that of scrub typhus, many SFGR infections, and *O. tsutsugamushi* and SFGR co-infected cases, may, therefore, be un- or mis-diagnosed.

According to phylogenetic analysis, *Rickettsia* species TwKM01 was most closely related to *R. rhipicephali* in *R. sanguineus* ticks from Mississippi, strain R300 (98.9% and 98.9% identity for *gltA* gene and 17-kd genes, respectively) in *Haemaphysalis justakochi* ticks from Brazil, and *R. massiliae* (99.9% identity for the *gltA* gene) in *R. turanicus* ticks from France, followed by strain Bar 29 (98.1% identity for the *gltA* gene) in *R. sanguineus* ticks from Spain and *R. aeschlimanni* (99.8% identity for the *gltA* gene) in *Hyalomma marginatum* ticks from Morocco (Figures 4 and 5). Although *R. massiliae* can cause human disease, whether the other rickettsiae in this cluster also cause human disease remains to be further examined. In the *gltA* phylogenetic tree (Figure 4), the TwKM02 and TwKM03 strains belong to the *R. australis* cluster and *R. felis* URRWXCal2 cluster, respectively. The closest relatives of the TwKM02 seemed to be the geographically distinct *R. australis*, isolated from *Ixodes* sp. tick in Australia, and *R. akari* (97.3% identity for *gltA* gene) from *L. sanguineus* house-mouse mite in New York (Figure 4). *R. australis* and *R. akari* are the causative agents of Queensland tick typhus and Rickettsial pox, respectively. TwKM02 may, therefore, be the unique mite-borne rickettsia. The relationship between TwKM02 strain and Leptotrombidial chigger mites shall be further studied from unfed chiggers and free-living adult mites. Finally, the phylogenetic position of the TwKM03 was located in the cluster with *R. felis* URRWXCal2. *Rickettsia felis* is an emerging pathogen responsible for flea-borne spotted fever and has been reported in various countries worldwide, including the United States, Mexico, Brazil, Germany, Spain, Peru, New Zealand, United Kingdom, Yucatan, and France. In Asia, Parola and others reported the first case of *R. felis* infection in Japan and South Korea. Ticks were suggested to carry *R. felis*–like rickettsia (Genotype V) in Japan recently, because it could be detected in *Haemaphysalis kitasatoe, H. flava*, and *I. ovatus* ticks. In 2006, a case report and serologic evidence of *R. felis* infection in humans was suggestive of human disease in Mexico and Spain. We also detected *Rickettsia* species TwKM03 in a broad range of arthropod hosts including *I. granulatus* ticks, Mesostigmata mites, and *L. deliense* chiggers. Because *Rickettsia* species TwKM03 was not detected from fleas, whether the *Rickettsia* species TwKM03 is certainly *R. felis* remains to be further clarified. It is not certain whether *Rickettsia* species TwKM03 and Genotype V (temporarily) propagated in the tick or arthropods such as the cat flea were from the infected animal or inherited through transovarial transmission as in the case of the cat flea. *Rickettsia* species TwKM02 detected in Leptotrombidial chigger mites might be the same rickettsia that originally come from rodents infected by ticks. Elucidating the exact pattern of arthropod host switching depends on further studies of hosts and additional *Rickettsia* species and strains for a more precise analysis. It is therefore important to examine more non–tick-borne rickettsiae and see their grouping in phylogenetic analyses. Previous studies indicated that there was no strict co-evolution between species of genus *Rickettsia* and the species of tick vectors. Rather, SFGRs were initially associated with *Rhipicephalus* ticks and subsequently radiated into other arthropod hosts multiple times. Taken together with the global distribution of *R. felis*, for example, the relationship of the SFGRs to their arthropod hosts may be more important than to their geographical locations. We suggest that further testing with additional gene segments, such as *ompA*, *ompB*, and gene D, is needed.

The RFLP procedure of PCR-amplified fragments of the *gltA* gene is a sensitive and easy-to-perform approach to identify rickettsiae. The endonuclease MacII was therefore chosen for genotyping of the SFGR strains. The 15 *gltA*–positive samples were grouped into three clusters, *Rickettsia*...
species TwKM01, TwKM02, and TwKM03, and had the same predicted restriction patterns as did the *R. rhipicephali*, *R. australis*, and *R. felis* URRWXCalc clusters, respectively (Figures 2 and 3). This result also corresponded well with the phylogenetic analysis of their gltA genes (Figure 4). Therefore, this method may be used for the rapid genotyping of SFGRs from patients, rodents, and arthropod vectors and may help to clarify the distribution of each genotype in the endemic area.

Three *Rickettsia* species TwKM01 isolates were cultured in L929 cells. However, no cytopathogenic effect was observed in any of the inoculated cell cultures and often a few scattered cells were infected with rickettsiae, even after 10 days of culture (Figure 6). Conditions for *Rickettsia* species TwKM01 cultivation need further testing and refinement. This result indicates that the *Rickettsia* species TwKM01 strain might have a low infection rate or slow growth rate; otherwise, anti-SFG human serum (PanBio) could cross-react with the TwKM01 strain.

In conclusion, we described three molecular SFGR, *Rickettsia* species TwKM01, TwKM02, and TwKM03, detected in Taiwan (Table 1). This finding raises the possibility of double infection of spotted fever and scrub typhus among the chigger mite population in Taiwan, although the ecologic properties between SFGRs and chiggers and affectivity to human patients should be further studied. Based on the phylogenetic analysis, three Taiwanese strains were not the most closely related to one another. Newly isolated *Rickettsia* species TwKM01 rickettsiae will be very useful for the development of serologic tests to determine their clinical spectrum, prevalence, and distribution in Taiwan.

Received February 14, 2007. Accepted for publication August 2, 2007.

Acknowledgments: We thank the rodent-related infectious disease surveillance team of the Institute of Preventive Medicine, National Defense Medical Center, Republic of China, for the collection of field samples, and Li-H-Jeng Tarn and Chia-Tsui Yeh for animal manipulations.

Financial support: This research was funded by the Ministry of National Defense of the Republic of China and partially supported by Grant NSC 91-2314-B-016-039 from the National Science Council, Taiwan.

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REFERENCES


