




Article

Seroepidemiological Study of Spotted Fever Group Rickettsiae and Identification of a Putative New Species, *Rickettsia* sp. Da-1, in Gongliao, Northeast Taiwan

Tsai-Ying Yen ¹, Hsi-Chieh Wang ¹, Yin-Chao Chang ², Chien-Ling Su ³, Shu-Fen Chang ³, Pei-Yun Shu ^{3,*} and Kun-Hsien Tsai ^{1,4,*}

¹ Institute of Environmental and Occupational Health Sciences, College of Public Health, National Taiwan University, Taipei 100025, Taiwan; d00849002@ntu.edu.tw (T.-Y.Y.); sjwang1019@gmail.com (H.-C.W.)

² Dr. Enjoy's Clinic, Gong-Liao District, New Taipei City 228003, Taiwan; chapter767474@gmail.com

³ Center for Diagnostics and Vaccine Development, Centers for Disease Control, Ministry of Health and Welfare, Taipei 115210, Taiwan; sue@cdc.gov.tw (C.-L.S.); vivi@cdc.gov.tw (S.-F.C.)

⁴ Department of Public Health, College of Public Health, National Taiwan University, Taipei 100025, Taiwan

* Correspondence: pyshu@cdc.gov.tw (P.-Y.S.); kunhtsai@ntu.edu.tw (K.-H.T.); Tel.: +886-2-26531372 (P.-Y.S.); +886-2-33668103 (K.-H.T.)



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Abstract: Tick-borne spotted fever group (SFG) rickettsioses were neglected in Taiwan. The study reported a seroepidemiological survey of SFG rickettsiae in residents in Gongliao District, Northeast Taiwan. Blood samples were examined for antibodies against SFG rickettsiae by enzyme-linked immunosorbent assay and immunofluorescence assay. Risk factors were assessed using logistic regression. Ticks parasitizing dogs were collected within a 2 km radius from the houses of seropositive participants, and PCR was performed to detect possible tick-borne pathogens. Of 1108 participants, 75 (6.8%) had antibodies against SFG rickettsiae. Residents were more likely to be seropositive if they were older than 65 years, recruited by Dr. Enjoy's Clinic, or resided in Jilin village. A total of 184 ticks including 5 species (*Rhipicephalus sanguineus*, *Rhipicephalus haemaphysaloides*, *Dermacentor auratus*, *Haemaphysalis hystricis*, *Haemaphysalis ornithophila*) were collected. *Rickettsia* spp. were detected in 6.5% (12/184) of ticks. *Rickettsia* sp. TwKM01 was found in 6 *R. sanguineus* and 4 *R. haemaphysaloides*; while *Rickettsia* sp. TwKM03 was identified in 1 *R. sanguineus*. Moreover, gene-based pairwise analysis indicated identification of a putative new species, *Rickettsia* sp. Da-1, in *D. auratus*. These findings provided evidence of SFG rickettsiae infection in ticks and suggested SFG rickettsiae exposure in the residents.

Keywords: spotted fever group rickettsiae; seroprevalence; *Dermacentor auratus*; *Rickettsia* sp. Da-1

1. Introduction

Rickettsiae are obligate intracellular Gram-negative bacteria belonging to the order Rickettsiales [1]. Genus *Rickettsia* was classified into 3 groups: spotted fever group (SFG) rickettsiae, typhus group (TG) rickettsiae, and scrub typhus group based on serology [2]. Later, the scrub typhus group was designated as a new genus, *Orientia* [3]. Further genetic evidence has suggested the addition of a transitional group (TRG) along with the ancestral group (AG) rickettsiae [4]. SFG rickettsiae and AG rickettsiae are primarily transmitted by ticks; while TG rickettsiae and TRG rickettsiae are associated with fleas, chiggers, or lice [5].

In Taiwan, the notifiable rickettsial diseases cover scrub typhus, epidemic typhus, and murine typhus. Scrub typhus and murine typhus led to 300–500 and 20–60 confirmed cases each year, respectively; while epidemic typhus has not been documented since World War II [6]. The fact that only 13.1–19.9% of the blood samples sent to the Taiwan CDC for laboratory diagnosis of scrub typhus were actually positive for *Orientia tsutsugamushi*

infection suggests other microorganisms were involved in causing the illness [6]. Indeed, human cases of granulocytic anaplasmosis (11/274, 4.0%) has been demonstrated from these samples, and cases of human monocytic ehrlichiosis were recently identified [7–9]. Infection of *Rickettsia felis*, a *Rickettsia* now belonging to the TRG, has also been reported in 0.7–3.3% of patients with fever of unknown origin [10–12]. SFG rickettsiae infection shares similar clinical features with scrub typhus [1]; however, only one record of imported *Rickettsia africae* infection has been described in Taiwan [13]. Contradictorily, SFG rickettsiae were common in the field. *Rickettsia conorii* has been found in 7.1–50.0% of *Ixodes granulatus*, 0.9% (1/109) of *Rhipicephalus haemaphysaloides*, and 1.3% (1/80) of *Stilvalius aporus* fleas in Central and East Taiwan and on the off-shore islands [14,15]. *Rickettsia japonica* was detected in *Haemaphysalis bandicoda* (7/10, 70.0%) and *S. aporus* (2/80, 2.5%) in South and East Taiwan, as well as *Rickettsia rickettsii* in *I. granulatus* (1.1%–9.4%) and *R. haemaphysaloides* (4/109, 3.7%) on the offshore islands [14,15]. *Rickettsia helvetica* and *Rickettsia monacensis* were identified in *Ixodes columnae* (8/17, 47.1%) and *Ixodes nipponensis* (1/1, 100%) collected from birds [16]. *Rickettsia parkeri*-like species were found in *I. granulatus* in Kinmen (12/247, 4.9%) [17]. Infection of small mammals by *R. conorii*, *R. japonica*, *R. rickettsii*, and *Rickettsia raoultii* were demonstrated with serological or molecular evidence [14,18,19]. Besides, a growing number of new species, including *Rickettsia* sp. TwKM01, *Rickettsia* sp. IG-1, *Rickettsia* sp. RR01, *Rickettsia* sp. TwKM02, and *Rickettsia* sp. TwKM03, have been identified in *R. haemaphysaloides*, *S. aporus*, *I. granulatus*, *Rhipicephalus sanguineus*, and *Leptotrombidium deliense* chigger mites, in Central and East Taiwan and on the offshore islands [20–22]. The diverse and widespread of *Rickettsia* spp. should serve as a warning sign to human infection of rickettsial diseases. Thus, we have reason to believe that SFG rickettsiae infection is unrecognized and underestimated in Taiwan.

Ticks are important medically not only because they are ectoparasites of vertebrates, but they can transmit various pathogens [23]. Approximately 896 species of ticks belonging to 3 families, Argasidae, Ixodidae, and Nuttalliellidae, were recognized worldwide [24]. Thirty-nine tick species occur in Taiwan [16,25–27]. Parasitic genera such as *Anaplasma*, *Babesia*, *Borrelia*, *Ehrlichia*, and *Rickettsia* have been detected in *R. haemaphysaloides*, *Haemaphysalis ornithophila*, *R. sanguineus*, *I. granulatus*, *Ixodes ovatus*, *Ixodes turdus*, *Haemaphysalis flava*, *I. columnae*, and *I. nipponensis*, suggesting the risk of infection by most of the identified tick-borne pathogens in people in Taiwan [16,28–30]. However, tick-borne diseases were reported scarcely [7–9,31,32]. In fact, most of the surveys were conducted on off-shore islands, and information about regional abundance of tick species was limited. Moreover, recent discovery of severe fever with thrombocytopenia syndrome virus in *Rhipicephalus microplus* as well as the first human case highlighted the importance of continuous surveillance of ticks and tick-borne diseases [33,34].

An early survey conducted in Tainan detected prevalence of 3.5–4.4% for antibodies against SFG rickettsiae, and another research also conducted in the southern Taiwan showed a seropositive rate of 2.9% (12/413) for SFG rickettsiae antibodies in patients suspected with Q fever, scrub typhus, murine typhus, leptospirosis, and dengue fever [11,35]. In order to clarify the extent of human SFG rickettsiae infection, here we report a cross-sectional study of the seroprevalence of SFG rickettsiae in residents of Gongliao District in New Taipei City, Taiwan. Potential tick vectors were collected, and infection of pathogens were screened to evaluate the risks of tick-borne diseases in the region.

2. Results

2.1. Demographics of the Participants

A total of 1108 blood samples were collected during January to December in 2008. The participants were aged 12 to 104 (median age 64 years), and the average age of subjects from Dr. Enjoy's Clinic was slightly older than those from the other two sources ($p < 0.01$). More than half of the participants were females (571/1108, 51.5%). Most samples from males were collected from patients visiting the group practice center ($p < 0.01$). The study population resided in 11 villages of Gongliao District. However, majority of the subjects

enrolled by the group practice center inhabited the northeastern Gongliao and the coastal villages; while subjects enrolled by Dr. Enjoy's Clinic mostly lived in the southwestern Gongliao (Table 1).

Table 1. Characteristics of study population.

Variables	Annual Health Exam (n = 260)	Patients Visiting the Group Practice Center (n = 557)	Patients Visiting Dr. Enjoy's Clinic (n = 291)	p Value
Gender				<0.01
Male	104	327	106	
Female	156	230	185	
Age (mean \pm SD)	55.1 \pm 15.2	58.2 \pm 20.4	63.3 \pm 16.1	<0.01
Village				<0.01
Gongliao (GL)	12	24	106	
Jilin (JL)	1	9	24	
Shuangyu (SY)	10	29	41	
Longgang (LG)	0	4	46	
Longmen (LM)	8	25	14	
Fulong (FL)	23	65	21	
Renli (RL)	83	137	18	
Zhenli (ZL)	78	149	11	
Fulian (FN)	14	31	6	
Meifeng (MF)	29	38	3	
Hemei (HM)	2	46	1	

2.2. Serology

Of 1108 serum samples, 118 (118/1108, 10.6%) were positive for SFG rickettsiae antibodies by enzyme-linked immunosorbent assay (ELISA). IgG against *R. rickettsii* was detected in 77 (77/1108, 6.9%) samples by immunofluorescence assay (IFA), and 75 of them were positive by both IFA and SFGR ELISA, resulting in a seropositive rate of 6.8% (75/1108) for SFG rickettsiae. Besides, 68 (68/1108, 6.1%) samples had antibodies reacting to *R. conorii* (Table 2). The highest prevalence of IgG against SFG rickettsiae was identified in the participants from Jilin village (Figure 1).

Table 2. Residents in Gongliao District with antibodies against spotted fever group rickettsiae, typhus group rickettsiae, and scrub typhus examined by SFGR ELISA and IFA.

	SFGR ELISA (n = 118)	IFA				
		<i>R. rickettsia</i> (n = 77)	<i>R. conorii</i> (n = 68)	<i>R. typhi</i> (n = 45)	<i>O. tsutsugamushi</i> (n = 155)	<i>R. rickettsii</i> + <i>R. typhi</i> (n = 23)
SFGR ELISA	-	75	68	26	15	23
IFA						
<i>R. rickettsii</i>	75	-	62	23	5	-
<i>R. conorii</i>	68	62	-	24	9	23
<i>R. typhi</i>	26	23	24	-	5	-
<i>O. tsutsugamushi</i>	15	5	9	5	-	10

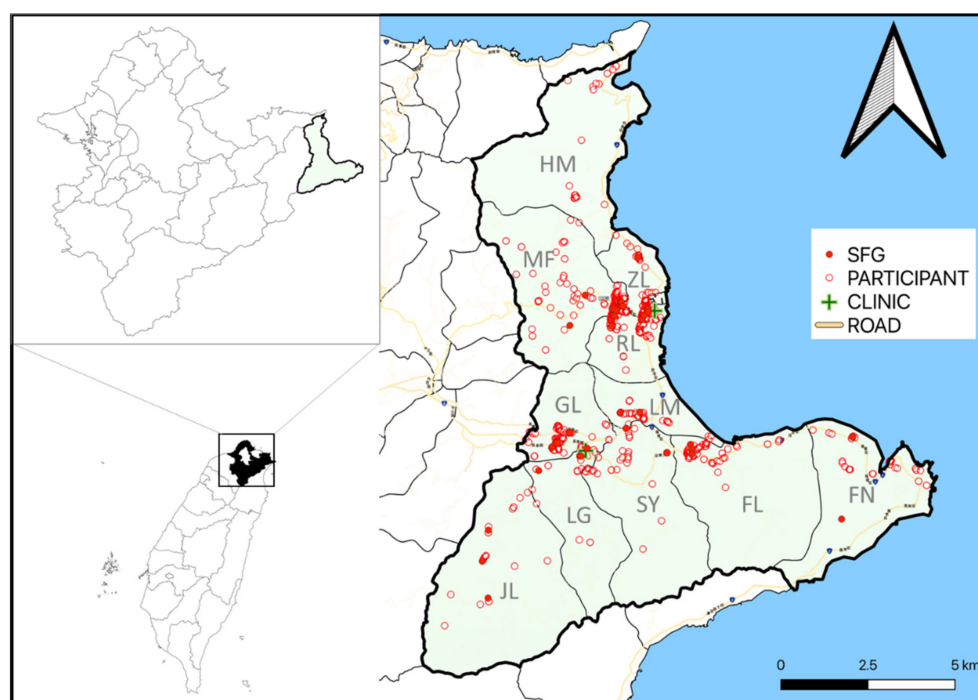


Figure 1. Location of samples positive for IgG antibodies against spotted fever group rickettsiae. GL: Gongliao Village; JL: Jilin Village; SY: Shuangyu Village; LG: Longgang Village; LM: Longmen Village; FL: Fulong Village; RL: Renli Village; ZL: Zhenli Village; FN: Fulian Village; MF: Meifeng Village; HM: Hemei Village.

Given that scrub typhus is the most reported rickettsial disease in Taiwan and cross-reactivity occurs between rickettsiae, antibodies to *R. typhi* and *O. tsutsugamushi* were examined for further comparison. Antibodies against *Rickettsia typhi* were found in 45 (45/1108, 4.1%) samples; while 155 (155/1108, 14.0%) samples were seropositive to *O. tsutsugamushi*. Forty-three sera reacted to more than one group of rickettsiae. Of them, 23 (23/1108, 2.1%) had antibodies against SFG rickettsiae (*R. rickettsii*) and TG rickettsiae; 5 (5/1108, 0.5%) had antibodies against SFG rickettsiae (*R. rickettsii*) and *O. tsutsugamushi*; 5 (5/1108, 0.5%) had antibodies against TG rickettsiae and *O. tsutsugamushi*; 10 (10/1108, 0.9%) reacted to SFG rickettsiae (*R. rickettsii*), TG rickettsiae, and *O. tsutsugamushi*.

2.3. Potential Risk Factors for SFG Rickettsiae Exposure

Univariable logistic regression revealed significantly positive association between SFG rickettsiae IgG seropositivity and older age, patients visiting either the group practice center or Dr. Enjoy's Clinic versus healthy individuals, living in Jilin village, and the job of industrial laborer (Table 3). In multivariable logistic regression, seropositivity remained significantly associated with age ($p = 0.014$). Sera positive for IgG against SFG rickettsiae were 2.1 times more likely to be collected from participants ≥ 65 years-old (95% CI = 1.2–3.8) and 3.3 times more likely to be from inhabitants of Jilin village. Reactivity to multiple groups of rickettsiae was also positively associated with older age ($p = 0.009$) and patients of the group practice center (95% CI = 1.2–53.3) or Dr. Enjoy's Clinic (95% CI = 2.8–255.6).

2.4. Collection of Ticks

To further understand the transmission of SFG rickettsiae in the area, ticks were collected from dogs living within 2 kilometer radius from the participants having IgG against SFG rickettsiae. Of 72 dogs encountered, ticks were found on 14 dogs, giving an infestation rate of 19.4% (14/72). A total of 184 ticks were collected including 155 *R. sanguineus*,

24 *R. haemaphysaloides*, 1 *Dermacentor auratus* (MZ823781), 3 *H. hystricis* (MZ823778), and 1 *H. ornithophila* (MZ823776).

Table 3. Seroprevalence and logistic regression analysis of associated possible correlates for SFG rickettsiae exposure in subjects in Gongliao District, New Taipei City, Taiwan.

Variables	No. of Samples Tested	No. (%) of Positive Samples	Univariate Regression Analysis	Multiple Regression Analysis
			OR (95% CI)	OR (95% CI)
Gender				
Male	537	39 (7.3)	Reference	ND
Female	571	36 (6.3)	0.9 (0.5–1.4)	ND
Age				
<65 yr	564	21 (3.7)	Reference	Reference
≥65 yr	544	54 (9.9)	2.9 (1.7–4.8) ***	2.1 (1.2–3.8) *
Sampling site				
Annual health exam	260	10 (3.8)	Reference	Reference
Group practice center	557	41 (7.4)	2.4 (1.1–5.2) *	1.7 (0.7–3.9)
Dr. Enjoy's Clinic	291	24 (8.2)	3.4 (1.5–7.5) **	2.2 (0.8–5.8)
Village				
Gongliao (GL)	142	9 (6.3)	Reference	Reference
Jilin (JL)	34	7 (20.6)	3.8 (1.3–11.2) *	3.3 (1.1–10.1) *
Shuangyu (SY)	80	8 (10.0)	1.6 (0.6–4.4)	1.5 (0.5–4.1)
Longgang (LG)	50	5 (10.0)	1.6 (0.5–5.2)	1.4 (0.4–4.5)
Longmen (LM)	47	4 (8.5)	1.4 (0.4–4.7)	1.6 (0.4–5.7)
Fulong (FL)	109	7 (6.4)	1.0 (0.4–2.8)	1.2 (0.4–3.7)
Renli (RL)	238	11 (4.6)	0.7 (0.3–1.8)	0.9 (0.3–2.7)
Zhenli (ZL)	238	10 (4.2)	0.6 (0.3–1.6)	0.8 (0.3–2.4)
Fulian (FN)	51	4 (7.8)	1.3 (0.4–4.3)	1.9 (0.5–7.3)
Meifeng (MF)	70	6 (8.6)	1.4 (0.5–4.1)	1.6 (0.5–5.3)
Hemei (HM)	49	4 (8.2)	1.3 (0.4–4.5)	1.8 (0.5–7.2)
Occupation				
NA	239	22 (9.2)	Reference	Reference
Agricultural worker	67	6 (9.0)	1.0 (0.4–2.5)	0.7 (0.2–1.9)
Housemaker	331	29 (8.8)	0.9 (0.5–1.7)	0.9 (0.4–1.8)
Industrial laborer	191	7 (3.7)	0.4 (0.2–0.9) *	0.6 (0.2–1.5)
Businessman	57	0 (0.0)	0.0 (0.0)	0.0 (0.0)
Government official	27	1 (3.7)	0.4 (0.0–2.9)	0.5 (0.1–4.4)
Teacher	7	1 (14.3)	1.6 (0.2–14.3)	2.7 (0.3–25.9)
Armed force occupation	1	0 (0.0)	0.0 (0.0)	0.0 (0.0)
Student	15	1 (6.7)	0.7 (0.1–5.6)	0.8 (0.1–7.2)
Other	173	8 (4.6)	0.5 (0.2–1.2)	0.6 (0.2–1.4)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; OR: odds ratio; CI: confidence interval; NA: not available; ND: not done.

2.5. Molecular Findings in Ticks

Rickettsia spp. were detected in 1 (1/1, 100%) *D. auratus*, 7 (7/155, 4.5%) *R. sanguineus*, and 4 (4/24, 16.7%) *R. haemaphysaloides*. The *gltA* amplicons (381 bp) from 6 *R. sanguineus* and 4 *R. haemaphysaloides* were identical to those of *Rickettsia* sp. TwKM01 (AY445819), 99.7% similar to *Rickettsia* spp. from India (MN463671.1, MN463666.1, MN557215.1–MN557217.1, MN557220.1–MN557224.1), and 99.2% similar to *Rickettsia massiliae* (KY640405.1) (Table 4). The *ompA* amplicons (1,073 bp) were identical to those of *Rickettsia* sp. TwKM01 (EF219467.1), 99.5% similar to *Rickettsia rhipicephali* (CP003342.1), and 99.4% similar to *R. massiliae* (U83444.1). The PCR product of *gltA* from 1 *R. sanguineus* was identical to *Rickettsia* sp. TwKM03 (AF540555), *R. felis* (GQ329873.1), and *Rickettsia* sp. RCF01 (GU056201.1). However, the amplified rickettsial fragments from *D. auratus* were relatively close to the sequences of *R. raoultii* and “*Candidatus Rickettsia laeensis*” [36] (Figure 2). Indeed, while the sequences from 5' end of *ompA* were 100% identical to the “*Candidatus R. laeensis*”

Table 4. Detection of *Rickettsia* spp. in ticks parasitizing dogs in Gongliao District, New Taipei City, Taiwan.

Tick Species (Accession No.)	No. Ticks (Female, Male, Nymph)	<i>Rickettsia</i> spp.	
		Positive Rate %(Positive/Tested)	Accession No.
<i>Dermacentor auratus</i> (MZ823781)	1 (1, 0, 0)	100.0 (1/1)	MZ869826
			MZ869827
			MZ869828
			MZ869829
			MZ869830
<i>Haemaphysalis hystrix</i> (MZ823778)	3 (2, 1, 0)	0.0 (0/3)	
<i>Haemaphysalis ornithophila</i> (MZ823776)	1 (0, 1, 0)	0.0 (0/1)	
<i>Rhipicephalus sanguineus</i>	155 (52, 37, 66)	4.5 (7/155)	AY445819 ¹
			AF540555 ¹
			EF219467.1 ¹
<i>Rhipicephalus haemaphysaloides</i>	24 (4, 7, 13)	16.7 (4/24)	AY445819 ¹
Total	184	6.5 (12/184)	

(A)

Rickettsia africae (U43790.1)
Rickettsia parkeri (U43802.1)
Rickettsia sibirica (U43807.1)
Rickettsia honei (U43809.1)
Rickettsia slovaca (U43808.1)
Rickettsia rickettsii (U43804.1)
Rickettsia conorii (U43806.1)
Rickettsia japonica (U43795.1)
Rickettsia heilongjiangensis (AF179362.2)
Rickettsia raoultii (MH532249.1)
Rickettsia amblyommatis (MF034496.1)
***Rickettsia* sp. Da-1 (MZ689827)**
Candidatus Rickettsia laeensis (MT321619.1)
Rickettsia aeschlimannii (U43800.1)
Rickettsia massiliae (MH532237.1)
Rickettsia rhipicephali (U43803.1)
Rickettsia montana (U43801.1)
Rickettsia australis (AF149108.1)
Rickettsia felis (AY279962.1)
Rickettsia akari (L01461.1)

0.20

(B)

Rickettsia mongolotimonae (AF123715.1)
Rickettsia africae (AF123706.1)
Rickettsia conorii (AF123721.1)
Rickettsia honei (AF123724.1)
Rickettsia rickettsii (X16353.1)
Rickettsia heilongjiangensis (AY260451.1)
Rickettsia japonica (AF123713.1)
***Rickettsia* sp. Da-1 (MZ689829)**
Rickettsia raoultii (EU036984.1)
Rickettsia massiliae (AF123714.1)
Rickettsia rhipicephali (AF123719.1)
Rickettsia felis (AF182279.2)
Rickettsia akari (AF123707.1)
Rickettsia australis (AF123709.1)
Rickettsia asiatica (DQ110870.1)
Rickettsia helvetica (AF123725.1)
Rickettsia bellii (AY970508.1)

0.050

(C)

Rickettsia slovaca (U59725.1)
Rickettsia sibirica (U59734.1)
Rickettsia africae (59733.1)
Rickettsia honei (U59726.1)
Rickettsia sp. IG-1 (EF219460.1)
Rickettsia rickettsii (U59729.1)
Rickettsia conorii (U59730.1)
Rickettsia heilongjiangensis (AB473662.1)
Rickettsia japonica (U59724.1)
Rickettsia raoultii (DQ365804.1)
Rickettsia aeschlimannii (DQ235776.1)
Rickettsia sp. Bar 20 (U59720.1)
Rickettsia rhipicephali (U59721.1)
***Rickettsia* sp. Da-1 (MZ689826)**
Uncultured Rickettsia sp. clone MVVJ16 (MH557219.1)
Rickettsia akari (U59717.1)
Rickettsia monacensis (EU965235.1)
Rickettsia tamurei (AB812551.1)
Rickettsia helvetica (KJ310588.1)
Rickettsia asiatica (AF34901.1)
Rickettsia bellii (AY362703.1)

0.030

(D)

Rickettsia mongolotimonae (AF151725.1)
Rickettsia africae (AF151724.2)
Rickettsia conorii (AF163005.1)
Rickettsia rickettsii (AF163000.1)
Rickettsia honei (AF163004.1)
Rickettsia sp. IG-1 (EF219462.1)
Rickettsia montanensis (AF163002.1)
Rickettsia japonica (AF155055.1)
***Rickettsia* sp. Da-1 (MZ689830)**
Rickettsia raoultii (DQ365807.1)
Rickettsia rhipicephali (AF155053.1)
Rickettsia massiliae (AF163003.1)
Rickettsia sp. Bar 29 (AF155056.1)
Rickettsia asenbomensis (KY650700.1)
Rickettsia akari (AF213016.1)
Rickettsia felis (AF196073.1)
Rickettsia helvetica (AF163009.1)
Rickettsia asiatica (DQ110869.1)
Rickettsia prowazekii (AF200340.1)
Rickettsia typhi (AF18482.1)

0.030

Figure 2. Phylogenetic tree of *Rickettsia* spp. constructed based on (A) *ompA* (375 bp); (B) *ompB* (2653 bp); (C) *gltA* (357 bp); (D) *sca4* (2261 bp) sequences. Rickettsial sequences derived from *D. auratus* were compared with those closely related species retrieved from GenBank. The evolutionary relationships were inferred by Maximum Likelihood method with 1000 bootstrap replicates.

Table 5. Pairwise comparison of partial sequences from *gltA*, *ompA*, *ompB*, and *sca4* between amplicons from *D. auratus* and references in GenBank. Cut-off values for molecular species identification was included.

Gene	% Pairwise Nucleotide Sequence Identity to Closest Neighbors (Accession No.)	No. Matching Nucleotides/Total	Cutoff Values [37]
<i>gltA</i>	99.74% to Uncultured <i>Rickettsia</i> sp. clone MIVLW15/2017 (MN557219.1)	383/384	99.9%
	99.74% to Uncultured bacterium clone HHMJ7 (KC566999.1)	381/382	
	99.48% to <i>R. raoultii</i> isolate N42 (MN550897.1)	382/384	
	98.41% to “ <i>Candidatus R. laoensis</i> ” (KT753290.1)	124/126	
5' end of <i>ompA</i>	100.0% to “ <i>Candidatus R. laoensis</i> ” isolate MHS2019/12 (MT321619)	551/551	98.8%
	98.55% to “ <i>Candidatus R. laoensis</i> ” isolate MIVLW15/2017 (MK905251.1)	543/551	
	97.64% to <i>R. raoultii</i> isolate z164 (MH532249.1)	538/551	
3' end of <i>ompA</i>	98.49% to <i>Rickettsia</i> sp. RpA4 (AH009131.2)	3134/3182	
	98.49% to <i>R. raoultii</i> strain Marne (AH015609.2)	3134/3182	
	98.40% to <i>R. raoultii</i> isolate Tomsk (MK304548.1)	3132/3183	
	97.99% to <i>R. raoultii</i> strain Khabarovsk (CP010969.1)	4333/4422	
<i>ompB</i>	97.96% to <i>R. raoultii</i> strain IM16 (CP019435.1)	4332/4422	99.2%
	97.94% to <i>R. raoultii</i> strain Khabarovsk (DQ365798.1)	4334/4425	
	99.28% to “ <i>Candidatus R. laoensis</i> ” (KT753294.1)	1101/1109	
	98.01% to <i>R. montanensis</i> str. OSU 85-930 (CP003340.1)	2417/2466	
<i>sca4</i>	97.82% to <i>R. raoultii</i> isolate Tomsk (MK304550.1)	2418/2472	99.3%
	97.73% to <i>R. raoultii</i> isolate Nsk862 (MT253668.1)	2416/2472	
	98.80% to “ <i>Candidatus R. laoensis</i> ” (KT753292.1)	820/830	

3. Discussion

Here we report an extensive study of *Rickettsia* exposure in the northeastern part of Taiwan. The community-based seroepidemiological survey including 1108 residents in Gongliao District revealed seroprevalence of 6.8% (75/1108) for SFG rickettsiae, 4.1% (45/1108) for TG rickettsiae, and 14.0% (115/1108) for *O. tsutsugamushi*. Ticks were collected from dogs living near the seropositive participants to look for potential tick-borne pathogens. *Rickettsia* spp. were detected in 6.5% (12/184) of the ticks. One putative new species similar to *R. raoultii* and “*Candidatus R. laoensis*” was discovered in *D. auratus* according to the criteria for molecular identification of *Rickettsia* [37]. Whether these microorganisms lead to human diseases remains to be investigated.

Our study showed 20.0% (222/1108) of the serum samples collected from residents in Gongliao District reacted to at least one group of rickettsiae. Considering IFA of *R. rickettsii* as the gold standard, the sensitivity and specificity of SFG ELISA were 97.4% and 95.8%, respectively. Forty-three of 222 (19.4%) reactive samples were positive to more than one group of rickettsiae. Cross-reactivity has been known to occur between SFG rickettsiae and TG rickettsiae, especially IgG antibodies [38]. Titration of sera was supposed to be performed to reveal differences in antibody titers for the distinction between cross-reactivity [39]. However, the observed IgG titers were generally quite low (≤ 256), making comparison using titration unrealistic. Therefore, we were unable to determine whether the reactivity was caused by exposure to different groups of rickettsiae or the result of cross-reactions. Moreover, sera from patients with *R. felis* infection were shown to react to *R. rickettsii* and *R. conorii*, and *R. felis* has been proposed to be the major cause of cross-reactions between *R. typhi* and *R. conorii* [40,41]. A study detected *R. felis* infection in 21.6% of patients with rickettsioses in North Taiwan [12]. Hence, *R. felis* exposure was speculated to be responsible for some of the reactions although the current study did not include *R. felis* in the assay. Conversely, cross-reactions between SFG rickettsiae/TG rickettsiae and scrub typhus were less common. Only 5 samples reacted to both *R. rickettsii* and *O. tsutsugamushi*, and 5 samples reacted to both *R. typhi* and *O. tsutsugamushi*. The prevalence of IgG antibodies against SFG rickettsiae, TG rickettsiae, and multiple groups of rickettsiae was significantly associated with older age, implicating progressive exposure to

Rickettsia spp. Samples collected from Dr. Enjoy's Clinic tended to have higher seropositive rates probably due to the older average age of the patients. Moreover, a part of the patients from Dr. Enjoy's Clinic was receiving home-based medical care voluntarily provided by the clinic. These patients generally lived in remote areas and belonged to a disadvantaged minority. As shown in the risk analysis, inhabiting the southwest village, Jilin, was a risk factor for seropositivity.

Although some scenic spots in Gongliao District have become popular attractions for tourists, most parts of the district remained rural. Dogs shuttling back and forth between the fields and human houses, making them a bridge to wildlife as well as their ectoparasitic ticks. In this case, the dogs were considered as sentinels and ticks were collected from the dogs living near seropositive participants. Most collected ticks were the brown dog ticks (*R. sanguineus*) and *R. haemaphysaloides* (155 and 24, respectively), which was generally consistent with other study [42], but species such as *D. auratus*, *H. ornithophila*, and *H. hystricis* also appeared in the collection. *Rickettsia* sp. TwKM01 and *Rickettsia* sp. TwKM03 were detected in *R. sanguineus* and *R. haemaphysaloides*. Phylogenetic analyses revealed *Rickettsia* sp. TwKM01 was most similar to *Rickettsia rhipicephali* [21]. *Rickettsia* sp. TwKM03, a species close to *R. felis*, was first identified in *Leptotrombidium* chigger mites and widely distributed in *I. granulatus* in Hualien, Kinmen, and Matsu in Taiwan [21]. The species has also been found to infect *R. sanguineus* and *Ctenocephalides felis* collected from dogs in Brazil [43]. Moreover, the sequencing results indicated a putative new species, *Rickettsia* sp. Da-1, was identified in *D. auratus*. Pairwise comparison revealed the amplified fragments of *gltA* and *sca4* were 98.4% and 98.8% identical to "*Candidatus R. laoensis*", respectively, which were lower than the gene sequence-based criteria of identification (99.9% and 99.3%, respectively) despite the 100% similarity of sequences from 5' end of *ompA* [37]. The amplicons of *ompB* were highly similar to "*Candidatus R. laoensis*", but the reference sequence was only 1109 base pairs. "*Candidatus R. laoensis*" was first discovered in a pool of *Haemaphysalis* ticks in Laos [36]. Later the *Rickettsia* was observed in 63.6% (14/22 pools) of ticks (*Haemaphysalis bispinosa*, *H. flava*, *H. hystricis*, *Haemaphysalis longicornis*, *Dermacentor atrosignatus*, *D. auratus*, *D. taiwanensis*, *Dermacentor silvarum*) infesting wild boars in Southeast China. However, the study used only partial *ompA* for the screening for infection [44]. The tick host in our study, *D. auratus*, has not previously been documented in Taiwan, but our follow-up studies confirmed the continuous existence of the species on the island (unpublished data). *Dermacentor auratus* has been found to parasitize wild boar, cattle, deer, buffalo, and small mammals in other countries, and cases of human infestation were recorded [45,46]. According to a personal communication, a female residing next to Gongliao District attended a clinic for being attacked by *D. auratus*. Whether *D. auratus* ticks can transmit rickettsiae to humans or small mammals and maintain rickettsiae in nature remains to be studied. On the other hand, one of the limitations of the current study resulted from our method of tick collection. For example, *I. granulatus*, which is abundant and has been shown to carry *Borrelia* and *Rickettsia* [22,47], was left out along with many other ticks known to occur in Taiwan. However, *I. granulatus* mainly parasitizes small mammals and there is no record of it attacking humans in the Australasian Zoogeographic Region [48]. Considering the terrain, the animal contact history, and the less active lifestyle of the participants, ticks were collected only from dogs in the study. Further surveys of ticks using different collection methods would provide more information regarding ticks and tick-borne pathogens in the region.

Tick-borne rickettsioses have been considered as important infectious diseases in the Western World. For example, Rocky Mountain spotted fever and human granulocytic anaplasmosis are nationally notifiable diseases in the USA [49]. Thirteen EU countries have established a surveillance system at the national level and reporting of rickettsioses are mandatory [50]. In Asia, Japanese spotted fever is a notifiable infectious disease in Japan, with approximately 200–300 cases reported annually and once a seropositive rate of 45.1% in Okinawa [51]. New pathogenic species, such as *Rickettsia heilongjiangensis*, *R. helvetica*, and *Rickettsia tamurae*, and other species with unknown pathogenicity, such

as *Rickettsia asiatica* and “*Candidatus Rickettsia tarasevichiae*”, have been identified after *R. japonica* [52–56]. In South Korea, Thailand, Malaysia, and Laos, seroprevalence for SFG rickettsiae has been described as 16.2–19.9%, 0.8–4%, 42.5%, and 2.6% in patients with acute febrile illness, respectively [57–62]. The seroprevalence was reported as 1.7% and 10.4–20.4% in healthy populations in Vietnam and Indonesia, respectively [63,64]. Spotted fever is a common disease in China, and the seroprevalence has been demonstrated as high as 54.8% in the healthy population in Eastern China [65]. New species, including *R. heilongjiangensis* and *Rickettsia sibirica mongolotimonae*, have also been identified [37,66].

In Taiwan, tick-borne SFG rickettsioses are still poorly understood. Our findings confirmed the exposure of SFG rickettsiae, with a seropositive rate of 6.8% in residents of the northeastern part of the island. *Rickettsia* spp. and other endosymbionts were identified in ticks, implying the existence of transmission cycles. Although the pathogenicity of these rickettsiae remained uncertain, SFG rickettsiae infection should be considered in the differential diagnosis of fever of unknown origin in addition to scrub typhus. This work emphasized the risk of tick-borne rickettsioses. Residents as well as tourists should wear personal protection equipment or repellent when engaging in outdoor activities, and practicing tick control in dogs is advised. Further investigations will focus on the patients to detect and isolate SFG rickettsiae for the verification of etiological agents of febrile illnesses. Other tick-borne pathogens, such as severe fever with thrombocytopenia syndrome virus, will also be explored.

4. Materials and Methods

4.1. Study Setting and Human Subject

Gongliao District is a rural district located in the northeastern part of Taiwan. The district comprises an area of approximately 99.97 square kilometers which is divided into 11 villages. The population was 13,970 in 2008 with 73.6% of the inhabitants dwelling in Zhenli, Renli, Hemei, Fulong, Goungliao, and Fulian villages. The terrain is generally hilly. Facing the Pacific Ocean, Gongliao District is famous for its golden-sand beach, the Fulong beach, and other scenic settings as well as old hiking trails. Tourists come to the district for seafood or recreation activities, such as surfing, windsurfing, fishing, hiking, and the music festival.

Human subjects were recruited through 3 sources, including healthy individuals attending physical examinations in a group practice center, non-febrile patients visiting the group practice center, and patients visiting or receiving home-based medical care provided by a clinic. Residents of Gongliao District, which covered workers who had been staying there for over 3 months, were invited to participate in the study. Blood samples were collected via venipuncture after obtaining the subjects' consent and left to stand for 30 min at room temperature. Sera were separated by centrifugation, aliquoted, and carried back to the laboratory on ice. The samples were then kept frozen at -20°C until further analyses. Meanwhile, willing participants were asked to fill out paper-based questionnaires to provide demographic information for further risk analysis. All participants did not exhibit typical signs of acute infections at the time of sampling.

4.2. Serology

4.2.1. Screening of Sera by ELISA for SFG Rickettsiae Exposure

Sera were tested for the presence of IgG antibodies against SFG rickettsiae using Panbio Spotted Fever Group IgG ELISA (Panbio, Brisbane, Australia). The assay was performed with serum samples diluted in serum diluent to 1:100. The PANBIO units were determined by dividing the sample absorbance by the average absorbance of the cut-off calibrator provided and multiplying by 10. A PANBIO unit >11 indicated a positive result, suggesting presence of detectable IgG to SFG rickettsiae.

4.2.2. Detection of IgG against SFG Rickettsiae, TG Rickettsiae, and *O. tsutsugamushi* by IFA

IgG antibodies against SFG rickettsiae and TG rickettsiae in the serum samples were examined by a commercially available IFA kit containing antigens of *R. rickettsii* and *R. typhi* (IF0100G, Focus Technologies Inc, Cypress, CA, USA) according to the manufacturer's instructions. Antibodies against tick-borne SFG rickettsiae were further screened using *R. conorii* Substrate Slide (IF0104, Focus Technologies Inc, Cypress, CA, USA) [19]. Briefly, sera were diluted to 1:32 and applied to the wells. After 30 min of incubation at 37 °C, the slides were washed with PBS. Fluorescein isothiocyanate (FITC) conjugated goat anti-human IgG/A/M (ThermoFisher Scientific Inc., Camarillo, CA, USA) was then added. After incubation, the slides were washed, dried, and mounted. The results were read at a magnification of 400× with a fluorescence microscope (Leica Microsystem, Singapore) by two technicians independently. An antibody titer of $\geq 1:64$ was considered as a positive reaction.

Antibodies against *O. tsutsugamushi* were screened by indirect IFA using slides coated with whole-cell antigens of the Karp strain as described [67,68]. A titer $\geq 1:64$ was considered seropositive and indicated potential exposure to *O. tsutsugamushi*.

4.3. Collection of Ticks and Tick Species Identification

Ticks parasitizing dogs were collected within a 2 km radius from the houses of participants who tested positive for SFG rickettsiae antibodies during August to December, 2009. Ticks were removed by forceps or a tick twister after obtaining agreement from the dog owners and stored in 70% ethanol. Species identification was carried out by observing the taxonomic characteristics under a dissecting microscope [69–72]. Molecular identification targeting the mitochondrial 16S rDNA and COI was applied to nymphs whose morphological features were not fully developed and adults whose key characteristics were undistinguishable due to damages during removal or engorgement with blood for confirmative purpose [69,73,74]. However, the coding sequences of COI were not efficiently amplified in nearly half of the specimens by PCR. Therefore, molecular identification was primarily dependent on the amplicons of the 16S rDNA. In addition, the 16S rDNA of ticks other than *R. sanguineus* and *R. haemaphysaloides* were sequenced to provide further information for the genetic characterization.

4.4. Detection of Potential Tick-Borne Pathogens

The collected ticks were cut symmetrically into halves, and a half of each tick was used in DNA extraction with Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan). PCR was performed to detect potential pathogens carried by ticks. Identification of rickettsial infections was achieved by primers targeting *ompA*, *ompB*, *gltA*, and *sca4* as previously described [75–79]. PCR products were sent to Mission Biotech (Taipei, Taiwan) for Sanger sequencing in both forward and reverse directions.

4.5. Phylogenetic Analysis

Sequences of the amplicons were first examined with Seqman 7.1.0 (Lasergene, Madison, WI, USA). Then the sequences were separately aligned against those closely related species found in GenBank using the Clustal W application within BioEdit 7.2.0. Phylogenetic trees were constructed based on the alignment using a maximum likelihood inference with 1,000 bootstrap replicates in MEGA7 [80]. Novelty of *Rickettsia* sp. was determined by the sequence identities of the pairwise comparison of *gltA*, *ompA*, *ompB*, and *sca4* sequences with their closest related species [37].

4.6. Statistical Analysis

The demographic information and serological results of participants were inputted to a Microsoft Excel (Microsoft Corporation, Washington, DC, USA) datasheet for further processing. A Kruskal-Wallis test (non-parametric test) was done to compare the geometric ratios between groups with a significance level of 0.05. Associations between subject char-

acteristics and serological results were studied using univariable and multivariable logistic regression, using the odds ratios and 95% Confidence Interval (CI) as measurement. All variables were treated as categorical. Multivariable analysis included variables which were significantly associated with seropositivity in the univariable analysis. Effect modification and interaction were assessed for each covariate associated with seropositivity. Statistical analyses were performed using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA).

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The DNA sequences generated during this study are openly available in GenBank. Other primary data are available on request from the corresponding author. Certain data are not publicly available due to ethical concern.

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