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Parasitology

The prevalence of *Anaplasma platys* and a potential novel *Anaplasma* species exceed that of *Ehrlichia canis* in asymptomatic dogs and *Rhipicephalus sanguineus* in Taiwan

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ABSTRACT. Canine anaplasmosis is regarded as an infection by *Anaplasma platys* rather than zoonotic *Anaplasma phagocytophilum* in subtropical areas based on the assumption that the common dog tick species is *Rhipicephalus sanguineus*, which transmits *E. canis* and presumably *A. platys*. We investigated asymptomatic dogs and dog ticks from 16 communities in Nantou County, Taiwan to identify common dog tick species and to determine the prevalence of *Anaplasma* and *Ehrlichia* spp. Of total 175 canine blood samples and 315 ticks, including 306 *R. sanguineus* and 9 *Haemaphysalis hystricis*, 15 dogs and 3 *R. sanguineus* ticks were positive for *E. canis*, while 47 dogs and 71 *R. sanguineus* ticks were positive for *A. platys,* via nested PCR for 16S rDNA and DNA sequencing of selected positive amplicons. However, among the dogs and ticks that were positive to *A. platys* 16S rDNA, only 20 dogs and 11 ticks were positive to nested PCR for *A. platys* groEL gene. These results revealed the importance of searching for novel *Anaplasma* spp. closely related to *A. platys* in dogs and ticks. Seropositivity to a commercial immunochromatographic test SNAP 4Dx *Anaplasma* sp. was not significantly associated with PCR positivity for *A. platys* but with infestation by ticks carrying *A. platys* ($P<0.05$). Accordingly, *R. sanguineus* may be involved in transmission of *A. platys* but may not act as a reservoir of *E. canis* and PCR results for 16S rDNA could be a problematic diagnostic index for *A. platys* infection.

KEY WORDS: 16S rDNA, *Anaplasma platys*, *Ehrlichia canis*, groEL gene, *Rhipicephalus sanguineus*

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Bacteria in the genus *Anaplasma* is a tick-borne rickettsial organism characterized by a distinct cellular tropism and host range and causes various diseases among animals [6]. The *Anaplasma* species known to infect dogs are *A. platys* and *A. phagocytophilum*, which are transmitted by different tick species. *A. phagocytophilum*, the causative agent of human granulocytic anaplasmosis, is a zoonotic pathogen that causes pathological conditions, represented by polyarthritis, cytoplasmic inclusions in neutrophils and various hematological abnormalities as well as fever, anorexia, lethargy, and lameness, in a wide range of mammals, including dogs, horses, ruminants, and humans. In contrast, *A. platys* is a rare organism that exclusively infects platelets and periodically causes profound thrombocytopenia only in dogs [1]. The clinical signs of *A. platys* infection are mild or often unrecognizable, despite the severity of thrombocytopenia; however, more severe morbidity has been reported in some countries [4, 14]. In addition, because *R. sanguineus* also transmits *Ehrlichia canis*, *A. platys* infections often occur concomitantly with monocytic ehrlichiosis caused by *E. canis,* resulting in severe clinical manifestations in dogs. Compared with *A. phagocytophilum* and infections involving this organism, which have been vigorously studied worldwide as an important emerging disease, *A. platys* and the infections it causes have been studied only in endemic regions. Despite the difference between these two *Anaplasma* spp., the serological diagnosis of *A. platys* infections in dogs has relied on a commercial ELISA kit utilizing a cross-reaction by antibodies against *A. phagocytophilum* and *A. platys* [10, 35], partly due to the unavailability of isolated *A. platys* culture strains [20]. However, the use of this kit against these two species may limit the ability to reveal the true presence of zoonotic anaplasmosis in areas where *R. sanguineus* is common [29].

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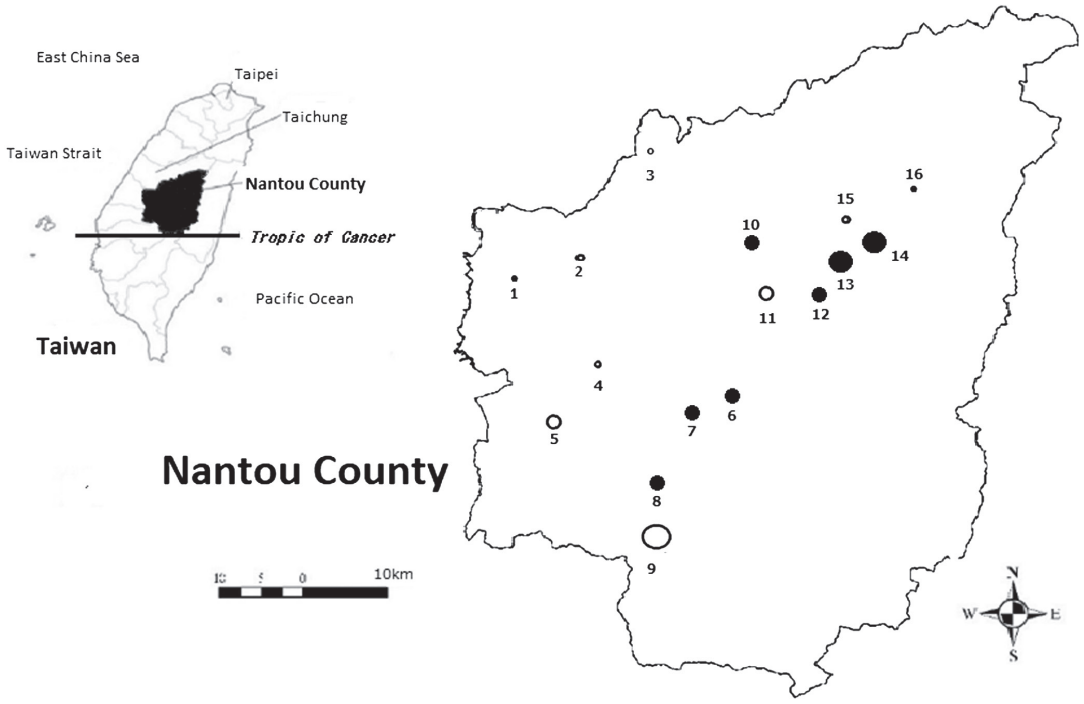

Fig. 1. Location of the study area, Nantou County, Taiwan. The 16 studied communities are represented by a circle simultaneously indicating both the degree of tick infestation (the bigger, the higher) and the local seroprevalence of *E. canis* and *Anaplasma* sp. infection determined via SNAP 4Dx (White: low seroprevalence. Black: high seroprevalence*). *low: the local seroprevalence of *E. canis* and/or *Anaplasma* sp. was lower than the average for the entire population. high: the local seroprevalence of *E. canis* and/or *Anaplasma* sp. was higher than the average for the entire population

A common vector for *A. phagocytophilum* is the *Ixodes ricinus* species complex, whereas the vector for *A. platys* is still unclear, although *R. sanguineus* has been suspected [1]. The geographic distribution of these tick species coincides with the distribution of either granulocytic or thrombocytic anaplasmosis in the region. *Ixodes ricinus* is distributed widely in Europe, causing tick-borne fever and related complications in ruminants and horses by transmitting *A. phagocytophilum*. *I. scapularis* plays a pivotal role in the spread of *A. phagocytophium* infections in domestic animals and humans from the upper Midwest to the northeastern states of the U.S.A. and Canada [19]. While the *Ixodes ricinus* species complex prefers cold, moist weather, *R. sanguineus* was originally a tropical species. Accordingly, the prevalence of *A. platys* infections has been reported more frequently in regions at lower latitudes, such as the state of Arizona in the U.S.A., Venezuela, Brazil, and Thailand [10, 16, 28, 31]. However, as *R. sanguineus* is highly adaptable to the environment, it is distributed worldwide [7], and the distribution of *A. platys* has reached northern temperate regions [17]. Moreover, the distribution of *I. scapularis* has shifted southward because of recent climate change [11]. To predict the risk of transmission of *Anaplasma* spp., such as *A. phagocytophilum* or *A. platys*, understanding the regional climate and the most likely tick species involved is of major importance. Information regarding how ticks and infectious organisms are distributed in a region aids in ascertaining the likelihood of exposure to these agents and will improve the diagnostic accuracy of these tick-borne diseases in domestic animals and humans.

In subtropical Taiwan, *R. sanguineus* has been recognized as the most common dog tick species [33]; therefore, *A. platys* and *E. canis* infections are of veterinary importance [5, 15]. However, the epidemiology of *A. platys* infections in dogs and ticks has not been studied. Moreover, one study identified rodents carrying *A. phagocytophilum* in Taichung city, which is located adjacent to Nantou County [23]. A previous study by our group revealed that the seroprevalence of canine anaplasmosis and ehrlichiosis differs across communities, ranging from virtually absent to more than 60%, despite the fact that local dogs are equally heavily- infested by ticks [38]. We hypothesized that this difference in local seroprevalence may be due to the differences in common dog tick species and the frequency of carriers present in each community. Hence, a cross-sectional, community-based study was conducted in Nantou County, Taiwan. The objectives of this study were to confirm the identity of the common tick species in local dogs and determine the prevalence of *Anaplasma/Ehrlichia* spp. known to infect dogs and ticks, in addition to comparing results between communities.

MATERIALS AND METHODS

Study area and population

Whole blood samples and infesting ticks from apparently healthy dogs were collected at the time of the annual rabies

Table 1. PCR results for dogs and ticks

Community ID	Number of sampled dogs and ticks		Number of dogs positive to SNAP 4Dx		Number of samples positive to PCR					
	Dogs	*R. sanguineus*	*Anaplasma* sp.	*Ehrlichia canis*	*Anaplasma platys* (16SrDNA and groEL)		*Ehrlichia canis*		*Anaplasma platys* 16SrDNA only	
					Dog	Tick	Dog	Tick	Dog	Tick
1	2	2	1	1	0	0	1	0	0	0
2	10	14	0	0	0	0	0	0	6	0
3	19	8	1	1	0	0	2	0	0	3 [a)]
4	8	4	0	0	0	0	0	0	2	0
5	0	6	NA	NA	NA	0	NA	0	NA	0
6	11	17	4	2	2	0	1	0	0	6
7	17	19	5	6	1	0	2	0	3	1
8	9	6	3	0	1	0	0	0	1 [a)]	0
9	11	26	0	0	6 [a)]	1	0	0	0	17 [a)]
10	7	1	2	1	0	0	0	0	0	0
11	26	56	1	0	1 [a)]	2 [a)]	1	0	7	4
12	14	29	9	4	3	1	2 [a)]	0	0	3
13	19	35	7	3	4 [a)]	2	4	2 [a)]	0	6
14	5	71	1	1	2	4 [a)]	1	1	0	18
15	14	9	2	1	0	0	1	0	8	2
16	3	3	1	0	0	1 [a)]	0	0	0	0
Total number	175	306 (282 [b)])	37 (21.1%)	20 (11.4%)	20 (11.4%)	11 (3.6%)	15 (8.6%)	3 (1%)	27 (15.4%)	60 (19.6%)

NA: not available. a) The PCR products were sequenced. b) The number of ticks whose host dogs also provided blood samples and demographic data.

vaccination campaign in urban and rural communities of Nantou County (23°58'N 120°58'E) in central Taiwan in the spring and fall of 2010 and 2011 [38] (Fig. 1). Prior to sample collection, the health condition of each dog was evaluated by a veterinarian and sample collection was conducted to the dogs whose owners gave consent to test their dogs. More than 80% of the county is covered with high mountains, and most of the studied communities are scattered in wooded mountainous areas. In Fig. 1, the studied communities are indicated by a circle simultaneously indicating the degree of tick infestation and the local seroprevalence of *E. canis* and *Anaplasma* sp. infection, as determined via the SNAP 4Dx test (IDEXX Laboratories, Westbrook, ME, U.S.A.). The seroprevalence of each community was compared with that of the entire study population, and each community was assigned to one of two groups, based on showing either a higher seroprevalence (high seroprevalence) or lower seroprevalence (low seroprevalence) than the study population: 21.1% for *Anaplasma* sp. and 11.4% for *E. canis* (Table 1). A total of 315 ticks and 175 dog blood samples were collected from 16 communities for this study. Among the 315 ticks, 291 were removed from 54 dogs that had provided blood samples (1 to 35 ticks per dog), and 24 ticks were collected without a blood sample from the host dog. After removal from the dogs, the ticks were preserved in tubes containing 70% ethanol and stored at room temperature prior to further analysis. Using a 23G sterile needle syringe, a maximum of 4 m*l* of peripheral blood was drawn from each dog into a sterile EDTA-containing vacutainer and maintained at 4°C during shipping to the laboratory for DNA extraction.

Hematological tests and DNA extraction

After arrival at the laboratory, a complete blood count and serological testing using SNAP 4Dx were performed. A 200 *µl* aliquot of whole blood that had been separated into a sterile tube and stored at −70°C prior to DNA extraction was analyzed with the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions.

Identification and DNA extraction from ticks

The ticks were morphologically identified under a light microscope using taxonomic keys from Walker *et al.* [34] and Teng and Jiang [32]. Each tick was treated as a single specimen. The tick was cut along the dorsomedial plane into bilaterally symmetrical right and left halves using a new sterile surgical blade. The left half of the body was employed for DNA extraction with the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's recommendation, and the remaining half was stored in 70% alcohol. The body halves of fully engorged females were further cut into 2 to 3 pieces, and DNA was extracted from each piece. Nymphs were cut into halves, and both halves were used for DNA extraction.

Screening via polymerase chain reaction (PCR) and gene sequencing

The DNA samples from dog blood and ticks were screened via PCR for *Anaplasma* and *Ehrlichia* species known to infect dogs. For the detection of *E. canis*, *E. chaffeensis*, *E. ewingii*, and *A. platys*, nested PCR for 16S rRNA genes was performed, whereas for *A. phagocytophilum*, the msp2/p44 genes were amplified. Briefly, in a final volume of 25 *µl*, 5 *µl* of template DNA was first amplified using the outer primers ECC and ECB (universal primers for *Anaplasma* and *Ehrlichia* species) [9], in a

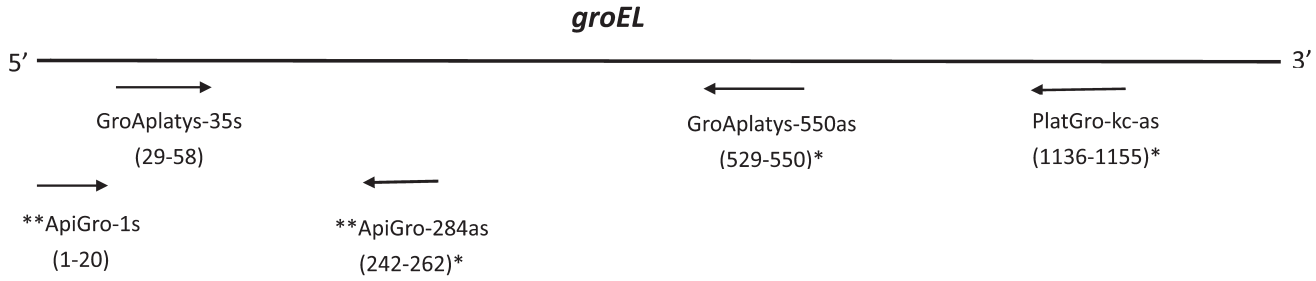

Fig. 2. Locations and orientations of the primers with respect to *A. platys* groEL gene. The numbers in each parenthesis represent the primer position relative to the nucleotide sequence of the *A. platys* groEL gene (GenBank accession number AF478129). *Reverse orientation. **The primer sequences of ApGro-1s and ApGro-284as were derived from the sites homology to multiple nucleotide sequences of *A. phagocyophilum* groEL genes (AF172158, EE473209, AF482760, KU519287).

reaction involving 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. For the nested amplification, we employed the specific primer pairs EPLAT 5 and EPLAT 3 [24] and CANIS and HE3 [2, 13] for *A. platys* and *E. canis*, respectively, and 2 *μl* of the primary PCR products was used as a template in a total volume of 25 *μl* (0.2 mM dNTPs, 2.5 U Taq DNA polymerase, 2.5X reaction buffer, and 10 pmol of each primer) employing an automated thermal cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for 35 cycles. For the detection of *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, 2 *μl* of the same primary PCR products was used as a template to perform nested amplification of 16S rRNA genes with specific primer pairs for each species (HE1 and HE3 [9] or EE72-ewingii and HE3 [8], respectively). To screen for *A. phagocytophilum*, PCR targeting the p44/msp2 gene was performed using MSP3F and MSP3R [21, 29, 39] and msp2 full-length F and R [21], with inner amplification using a newly designed primer pair (MSP2 In-F 5′-TATGGACTATCCGTGAGCAG-3′ and MSP2 In-R 5′-CCAAGTTTGAGCTTGTATGAAAG-3′). A negative control containing distilled water instead of a DNA template in the PCR mixture and positive controls containing previously sequenced and verified *A. platys*, *A. phagocytophilum*, *E. canis*, and *E. chaffeensis* DNA were included in each PCR run. The PCR products were electrophoresed on 2% agarose gels to confirm the expected size of the amplified nucleotide fragments, i.e., 385 bp for *A. platys*, 365 bp for *E. canis*, 389 bp for *E. chaffeensis*, 407 bp for *E. ewingii*, and 334 bp and 1,100 bp (745 bp for nested PCR) for *A. phagocytophilum*. To corroborate the PCR results, randomly selected PCR products showing a strong positive reaction were purified with the QIA quick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and DNA sequencing was performed directly on the purified PCR products using the fluorescence-labeled dideoxynucleotide technology of the ABI PRISM 3730 automated DNA sequencer.

PCR targeting the A. platys groEL gene

PCR targeting the *A. platys* groEL gene was conducted for further evaluation of the *A. platys* strain using an *A. platys*-specific primer pair for the groEL gene (GroAplatys-35s and GroAplatys-550as) [3]. Hemi-nested PCR for the *A. platys* groEL gene was also conducted using a newly designed primer (PlatGro-kc-as 5′-CCATCTGTGCTTTGATTTGG-3′). This PCR primer was derived from conserved regions on the basis of a multiple alignment of *A. platys* groEL sequences obtained from GenBank. Additional primers were designed to amplify both *A. platys* and *A. phagocytophilum* strains (ApGro-1s 5′-TAGTGATGAAGGAGAGTGAC-3′ and ApGro-284as 5′-TTCATTACCTTGTAGCCATCC-3′; 285 bp). These primer sequences were derived from conserved regions based on a multiple alignment of *A. platys* and *A. phagocyophilum* groEL genes. A diagram showing the primer locations and orientations was presented in Fig. 2. For each reaction, 2 *μl* of a DNA extract was used for PCR amplification in a total volume of 25 *μl*, containing 10 pmol of each primer. The reaction was visualized in a 2% agarose gel, and the positive PCR products (516 bp for inner amplicons and 1121 bp for outer amplicons) extracted from the gel were sequenced as described above.

Data analysis

A statistical analysis was performed with Systat 13® software (Systat Software, Inc., Chicago, IL, U.S.A.). Cohen's Kappa coefficient was used to evaluate the agreement between the serological test (SNAP 4Dx) and PCR results and is reported with the 95% confidence interval (CI). Pearson's χ^2 test was employed for univariate analysis of the categorical data. Hematological parameters were evaluated with *t*-tests for comparison of two independent groups to detect significant differences. Statistical significance was considered to be indicated by a *P*-value less than 0.05.

RESULTS

16S rDNA PCR results for DNA extracted from dog blood and ticks

Among the 315 ticks examined in this study, *Rhipicephalus sanguineus* was the dominant tick species, represented by 36 nymphs and 270 adults (122 females, and 148 males). *Haemaphysalis hystricis* was also found (a total of 9 ticks, including 2 engorged females, 6 males, and 1 nymph), but only in Communities 12 and 13 (Fig. 1). Most of the *R. sanguineus* nymphs (30/36 nymphs; 83.3%) were collected from mountainous communities visited in the fall (Communities 9 and 14). The PCR results for the

Table 2. Comparison of *A. platys* and *E. canis* nucleotide fragment sequences from this study with other *A. platys* and *E. canis* strains

a. *A. platys* 16S rDNA

A. platys strain	Host	GenBank accession number	Identity (%) [a]	Nucleotide positions [b]		
				215	320	321
This study (Y368)	dog	KY565478		T	-	-
Venezuela	dog	AF287153	99	C	A	A
Okinawa	dog	AY077619	100	T	-	-
Sommieres	dog	AF303467	100	T	-	-
Gzh981	dog	AF156784	100	T	-	-
Philippines	dog	JQ894779	100	T	-	-

a) The values are the percentage of sequence identity for a 348 bp sequence calculated from pairwise alignment.
b) The numbers represent the nucleotide position based on AF287153. The symbol (−) indicates a deletion.

b. *A. platys* groEL

A. platys strain	Host	GenBank accession number	Identity (%) [a]	Nucleotide positions [b]		
				341	368	895
This study (V493-2)	*Rhipicephalus sanguineus*	KY581623		A	G	G
Sommieres	dog	AY044161	99	G	A	G
Okinawa	dog	AY077621	99	A	A	G
Congo	*R. sanguineus*	AF478129	99	G	A	G
Brazil		EU516386	99	G	A	T
Philippines	*R. sanguineus*	JN121382	100	A	G	G

a) The values are the percentage of sequence identities for a 1,078 bp sequence calculated from pairwise alignment.
b) The numbers represent the nucleotide position based on AY044161.

c. *E. canis* 16S rDNA

E. canis strain	Host	GenBank accession number	Identity (%) [a]	Nucleotide positions [b]		
				132	205	358
This study (Y299)	dog	KY565476		T	G	A
Florida	dog	M73226	99	T	A	A
Lima	dog	DQ915970	100	T	G	A
Portugal	dog	EF051166	100	T	G	A
India	dog	GU182114	99	C	G	G
Taiwan	dog	DQ258496	100	T	G	A

a) The values are the percentage of sequence identities for 315 bp calculated from pairwise alignment.
b) The numbers represent the nucleotide position based on M73226.

dogs and ticks are summarized according to their respective communities (Table 1). In Table 1, only the PCR results from the 306 *R. sanguineus* specimens are shown because all nine *H. hystricis* were PCR negative for the various pathogens tested in this study. None of the samples examined in this study tested positive for *A. phagocytophilum*, *E. ewingii*, or *E. chaffeensis*.

Among the 175 dogs and 306 *R. sanguineus*, positive results of nested PCR for the 16S rRNA gene were obtained for *A. platys* in 47 dogs (26.9%) and 71 ticks (23.2%) and for *E. canis* in 15 dogs (8.6%) and only 3 ticks (1%). The origin of the sequenced positive amplicons is given in Table 1. The sequencing analysis (Table 2) indicated that the *E. canis*-positive nucleotide fragments were 100% identical to the *E. canis* strains from Taiwan (GenBank accession number: DQ258496), Lima (DQ915970), and Portugal (EF051166), while the *A. platys*-positive fragments were 100% identical to the *A. platys* strains from Okinawa (GenBank accession number: AY077619), Guangzhou (AF156784), the Philippines (JQ894779), and Sommieres, France (AF303467). No nucleotide variation was observed among the sequenced PCR amplicons.

Comparisons of positive test results between SNAP 4Dx and 16S rDNA PCR, and results of *A. platys* groEL gene PCR for dogs and ticks positive to *A. platys* 16S rDNA PCR.

Difference in seroprevalence among communities were depicted in Fig. 1. Among the 16 communities, 44% (7 communities) were classified as communities with low seroprevalence (range: 0–20% for *Anaplasma* sp. and 0–7.1% for *E. canis*), and 56% (9 communities) were classified as showing high seroprevalence (range: 28.5–64.3% for *Anaplasma* sp. and 15.8–50% for *E. canis*). Seropositivity for both *E. canis* and *Anaplasma* sp. was observed in most of the communities, except for Communities 8, 11 and 16, where the local dogs were only seropositive for *Anaplasma* sp., and for Communities 2, 4 and 9, where no dogs showed seropositivity to SNAP 4Dx testing (Table 1). However, contrary to our expectation, a number of dogs were positive to nested PCR for *A. platys* 16S rRNA gene regardless of the local seroprevalence. While there was moderate agreement between the test results from the 16S rRNA gene PCR and SNAP 4Dx analyses for *E. canis* (Kappa value: 0.525; 95% CI 0.315 to 0.735), the agreement

Table 3. Ticks carrying *A. platys* and their host dog infection status

Tick ID (Host dog ID- tick number)	Community ID	Stage	Host dog status		
			A. platys 16S rDNA	*A. platys groEL*	*E. canis 16S* rDNA
V437	9	female	+	+	–
V230-17	11	female	–	–	–
V230-29	11	female	–	–	–
V521-18	12	female	+	+	–
V481	13	female	+	+	+
V493-2	13	female	+	+	–
V496-1	14	male	+	+	–
V498-3	14	nymph	+	+	+
V498-5	14	nymph	+	+	+
V498-21	14	nymph	+	+	+
V261-2	16	male	–	–	–

between the *A. platys* 16S rRNA gene PCR assay and the SNAP 4Dx *Anaplasma* sp. results was very poor, at −0.060 (95% CI −0.199 <Kappa <0.078). Accordingly, PCR for the *A. platys* groEL gene [3] and hemi-nested amplification of this gene using a newly designed primer (PlatGro-kc-as) were conducted for all of the DNA samples that were positive for *A. platys* 16S rDNA. Among the 47 dogs and 71 ticks that were positive for the *A. platys* 16S rRNA gene, only 20 dogs and 11 ticks were positive for the groEL gene. The sequencing analysis of the positive amplicons showed them to be closest to the *A. platys* strain identified in *R. sanguineus* from the Philippines (JN121382), with 100% DNA identity (Table 2). Among the DNA samples negative for the *A. platys* 16S rRNA gene, none were positive for the *A. platys* groEL gene.

All of the DNA samples that were positive for the *A. platys* 16S rRNA gene but negative for the *A. platys* groEL gene (27 dogs and 60 ticks) were negative for the *Anaplasma* spp. groEL gene according to PCR using the newly designed primers ApGro-1s and ApGro-284as. A partial sequence of each gene (*E. canis* 16S rRNA and *A. platys* 16S rRNA and groEL genes) was submitted to GenBank under the accession numbers KY565476, KY565477, KY565478, KY565479, KY581623 and KY581624.

Definition of A. platys infection

The DNA samples that were positive for *A. platys* 16S rDNA according to PCR were divided into two groups based on the results of PCR for the *A. platys* groEL gene: one group positive for both the 16S rRNA and the groEL genes, while the other was positive for 16S rDNA but negative for the groEL gene. Because the nucleotide fragments amplified via nested PCR targeting the *A. platys* 16S rRNA gene were also 100% identical to unclassified *Anaplasma* spp., such as an *A. platys*-like *Anaplasma* sp. detected in goats in southern China (accession number: JN558821) and the *Anaplasmataceae* agent detected in brown brocket deer in Brazil (accession number: KF020572), we considered it possible that the nucleotide fragments may not conclusively verify the identity of the template DNA as *A. platys*. Accordingly, we defined *A. platys* as being confirmed by PCR for both the *A. platys* 16S rRNA and groEL genes, while positivity only for *A. platys* 16S rDNA indicated a probable unknown *Anaplasma* sp.

Distribution of dogs and ticks carrying E. canis, A. platys, and a probable unknown Anaplasma sp.

Among the 175 dogs examined, 8.6% (15/175) were PCR positive for *E. canis*, 11.4% (20/175) for *A. platys* and 15.4% (27/175) for an unknown *Anaplasma* sp., including 3 dogs co-infected with *E. canis* and *A. platys*. The *E. canis*-positive dogs came from Communities 1, 3, 6, 7, 11, 12, 13, 14 and 15 (Table 1). Although 67% (10/15 dogs) of the dogs carrying *E. canis* were detected via SNAP 4Dx, 13% (5 dogs) of the dogs carrying *E. canis* dogs in Communities 3, 7, 11 and 13 were negative according to SNAP 4Dx. Three ticks (1%) positive for *E. canis* as well as the 3 co-infected dog samples were collected from Communities 13 and 14. The three *E. canis*-positive ticks, including 2 fully engorged females and 1 engorged nymph, were removed from two dogs, both of which were infected with *E. canis*.

There was no significant association between the SNAP 4Dx results for *Anaplasma* sp. and *A. platys* subclinical infections (i.e., dogs positive for both the *A. platys* 16S rRNA and groEL genes) (P=0.076; χ^2 test); 60% (12/20 dogs) of *A. platys*-infected dogs were not detected by SNAP 4Dx. Notably, in Community 9, although 54% (6 of 11 dogs) of the local dogs were infected by *A. platys*, none of the dogs tested positive for *Anaplasma* sp. according to SNAP 4Dx. Interestingly, although 18 of the 26 ticks collected from Community 9 were removed from these 6 *A. platys*-positive dogs, almost all (17) of these 18 ticks were positive only for an unknown *Anaplasma* sp. Overall, there was a significant association between the SNAP 4Dx *Anaplasma* sp. results and the presence of infesting ticks carrying *A. platys* (P=0.008; χ^2). The geographical origins of the 11 *A. platys* PCR-positive ticks and the PCR results for their host dogs are summarized in Table 3. These *A. platys* positive ticks comprised 6 females, 2 males and 3 nymphs and were collected from 8 dogs from 6 communities. *A. platys* was identified in 2 females and 1 male tick removed from dogs that were negative for *A. platys* infection. Co-infection of *A. platys* and *E. canis* was not observed in the 11 ticks, despite the fact that 2 of the 8 host dogs were infected with both *A. platys* and *E. canis* (Table 3).

Communities with a high frequency of dogs positive for the unknown *Anaplasma* sp. were observed: Community 2 (60%: 6/10 dogs), Community 11 (26.9%: 7/26 dogs), and Community 15 (57.1%: 8/14 dogs) (Table 1). Overall, 60 ticks (19.6%)

from 9 communities were positive only for the *A. platys* 16S rRNA gene, including 15 nymphs, 22 females and 23 males, with a significantly higher prevalence being observed in nymphs (41.7% in nymphs vs. 18% in females and 15.5% in males, $P<0.05$). None of the dogs positive for the unknown *Anaplasma* sp. were positive for *Anaplasma* spp. according to SNAP 4Dx. The hematological profiles (such as platelet numbers and hematocrit values) of the dogs positive for the unknown *Anaplasma* sp. were within the normal range. The mean platelet number and mean hematocrit for this group were 256.4 × $10^3/\mu l$ (SD 105.5) ($P=0.603$, mean for dogs negative for *A. platys* 16S rDNA and dogs positive for *A. platys* groEL gene: 244.1 × $10^3/\mu l$, SD 133.9) and 42.96% (SD 7.2) ($P<0.05$, mean for dogs negative for *A. platys* 16S rDNA and dogs positive for *A. platys* groEL gene: 39.29%, SD 8.6), respectively, while the corresponding values for *A. platys*-infected dogs were 200.6 × $10^3/\mu l$ (SD87.7) ($P<0.05$, mean for non-infected dogs: 251.6 × $10^3/\mu l$, SD 133.271) and 34.64% (SD 6.6) ($P<0.05$, mean for non-infected dogs: 40.5%, SD 8.5).

DISCUSSION

This study was conducted based on the findings of our previous community-based study [38] to clarify the factors contributing to the marked differences in the seroprevalence of canine anaplasmosis among communities with equally high tick infestation levels. The results demonstrated that 8.6% (15/175 dogs) and 11.4% (20/175 dogs) of the dogs were carrying *E. canis* and *A. platys*, respectively, while *R. sanguineus*, the single dominant species in the study area, harbored *E. canis* at a frequency of only 1% (3/306 ticks) but *A. platys* at a frequency of 3.6% (11/306 ticks). The *A. platys* strain prevalent in this area appeared to belong to the same clone as an *A. platys* strain identified in the Philippines, with 100% identity. The results suggest that *R. sanguineus* plays a greater potential role in the transmission and maintenance of *A. platys* than as a reservoir of *E. canis*. A number of DNA samples positive for *A. platys* 16S rDNA unexpectedly tested negative for the *A. platys* groEL gene according to a hemi-nested PCR analysis, which resulted in an incomparable prevalence when calculated based on the 16S rRNA and groEL PCR results independently. One possibility for the occurred discrepancy might be because *Anaplasma* sp. that is not known to infect dogs or a potential novel *Anaplasma* sp. is prevalent in the study area. Dogs infected with this probable unknown *Anaplasma* sp. were not seropositive for *Anaplasma* sp. according to the SNAP 4Dx test and lacked the apparent hematological abnormalities accompanying *A. platys* infections; the prevalence was 15.4% (27/175 dogs) in dogs and 19.6% (60/306 ticks) in *R. sanguineus*, with some enzootic communities being observed.

The prevalence of *A. platys* in dogs according to PCR varies across geographical regions worldwide, e.g., 33% in Italy [30], 24.7% in Grenada [37], and 8.3% in Arizona [10]. A previous study reported a prevalence of 8.9% (4/45 pet dogs) in northern Taiwan [5], which was lower than the prevalence (11.4%) observed in our study. This may be due to differences in sampling, because most of the dogs (133/175) examined in the present work came from an environment with a high level of tick infestation, which has been linked to a high prevalence of *A. platys* in dogs [5, 18]. Our results confirmed this previous finding by revealing that subclinical *A. platys* infections were more frequently observed in communities with high tick infestation.

However, our study also revealed some controversial aspects regarding the detection and determination of *A. platys* infection. First, the target sequence for the PCR identification of *A. platys* may affect the *A. platys* prevalence observed via PCR considerably, as an 11.4% prevalence was determined based on PCR positivity for both the *A. platys* 16S rRNA and groEL genes, while a 26.9% prevalence was detected according to PCR positivity for only the 16S rRNA gene. The prevalences calculated based on the 16S rRNA and groEL PCR results were not comparable. As the reason for this, it is feasible to assume the presence of an unknown *Anaplasma* sp. that appeared to be carried by dogs and *R. sanguineus* was enzootic in the study area. The unknown *Anaplasma sp.* could be a novel species closely related to *A. platys*. This issue will be further discussed later in this section. Second, our findings also indicated the limited ability of SNAP 4Dx to detect *A. platys* infections. In contrast to a previous study [10], there was no significant correlation of the test results from the SNAP 4Dx test for *Anaplasma* sp. and the PCR test for *A. platys*. By using the new product, SNAP 4Dx Plus test, we have been experiencing similar discordant test results between PCR and serology (data not shown). Compared with the prevalence in the dog population examined in the previous study, in which *E. canis* infection was highly enzootic (greater than 36%), the PCR prevalence of *E. canis* observed in dogs in our study area was only 8.6%. It has been implied that persistent infection with *A. platys* may be more efficiently detected via SNAP 4Dx testing for *Anaplasma* sp. in the case of sequential or concurrent infections with *A. platys* and *E. canis* than in cases of infection by *A. platys* alone [12]. In addition, our results showed that infestation by *A. platys*-carrying ticks was positively associated with seropositivity for *Anaplasma* sp. according to SNAP 4Dx. Further work is required to address the mechanisms of seropositivity for *Anaplasma* sp. according to SNAP 4Dx in *A. platys* infections.

In our study, the frequency of *R. sanguineus* showing PCR positivity for *E. canis* was only 1% (3 of 306 ticks) (2 fully engorged females and 1 nymph); however, 8.6% (15/306) of the dogs were positive according to PCR. The prevalence of *E. canis* among ticks has been reported to be 2.0% in Malaysia [26]. In contrast to the results for *E. canis*, the frequency of *R. sanguineus* carrying *A. platys* was 3.6% (11/306 ticks), including 1 male and 2 female ticks that were removed from dogs not showing PCR positivity for *A. platys*. Notably, although 4 of the 11 *A. platys*-positive ticks were removed from 2 dogs infected with *A. platys* and *E. canis* concurrently, *E. canis* was not detected in these 4 ticks, despite the fact that one of them was an engorged female (Table 2). This may suggest that the results regarding *E. canis*-carrying ticks may not be due to the contamination of host dog blood. Because the tick population in this study did not include ticks in the questing stage, it is still unclear whether *R. sanguineus* could act as a natural reservoir or vector for *A. platys* transmission. It has been suggested that *R. sanguineus tropical* sp. is a competent vector for *E. canis* transmission, while that *R. sanguineus template* sp. is not [25]. Further studies on the vector competency of the *R. sanguineus* species complex are required to understanding the maintenance and transmission of *E. canis* and *A. platys* in Taiwan.

The prevalence of a novel *Anaplasma* sp. that is genetically close to *A. platys* has been reported in ruminants and deer from other regions of the world [22, 27, 36, 40]. However, to the best of our knowledge, this is the first report that a potential novel *Anaplasma* strain closely related to *A. platys* may be prevalent in dogs. The primer pair ApGro-1s and ApGro-284 was used in the *Anaplasma* groEL PCR with the aim of amplifying a broader range of *Anaplasma* groEL sequences as well as ruling out the possibility of *A. phagocytophilum*. GroEL sequences similar to other *Anaplasma* sp., but not to *A. platys* or *A. phagocytophilum*, have been detected in animals from which the 16S rDNA sequence close to *A. platys* was amplified simultaneously [40]. This study showed that the infected dogs exhibited no antibody reaction to *Anaplasma* sp. in SNAP 4Dx test, and the clinical significance of this organism appeared to not be associated with the hematological abnormalities commonly observed in *A. platys* infections. Further work is required to pursue the true identity of this unknown *Anaplasma* sp. through genetic and pathological analyses.

This study showed that the prevalence of *A. platys*, most closely related to an *A. platys* strain (JN121382) from the Philippines, predominated over that of *E. canis* in dogs and *R. sanguineus* in Nantou County of central Taiwan. This study also suggested that *R. sanguineus* plays a role as a vector and reservoir of *A. platys*, while its role as a reservoir of *E. canis* is suspect. PCR targeting the *A. platys* 16S rRNA gene should be avoided as a routine diagnostic method for *A. platys* infection because our results indicated that it is likely that an unknown *Anaplasma* sp. closely related to *A. platy is* prevalent among asymptomatic dogs and may affect the accurate detection of *A. platys* in this area. The unknown *Anaplasma* strain could be important for further identification. Further work is required to address the genetic analysis of *A. platys* and *Anaplasma* sp. prevalent in the study area as well as the acquisition process of *Anaplasma* sp. and *E. canis* by ticks in the natural environment.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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