First molecular detection of *Anaplasma phagocytophilum* in the hard tick *Rhipicephalus haemaphysaloides* in Taiwan

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Abstract

Anaplasma phagocytophilum is transmitted mainly by hard ticks and can cause potentially fatal granulocytic anaplasmosis in humans, but its occurrence in ticks in Taiwan has never been investigated although this pathogen has been detected in Taiwanese rodents before. Ticks collected from small mammals in Hualien, eastern Taiwan, were assayed for *Anaplasma* infections; infections of *Rickettsia* and Apicomplexa protozoans were also studied. Of the 270 individually assayed *Rhipicephalus haemaphysaloides* ticks, *A. phagocytophilum* was identified in a nymphal tick. Parasites most similar to *Anaplasma bovis*, *Rickettsia rickettsii*, *Rickettsia* sp. TwKM01, and at least seven apicomplexan species (including genera *Cryptosporidium*, *Hepatozoon*, and *Theileria*) were also identified. This study shows that *A. phagocytophilum* does occur in the hard tick in Taiwan, although whether *R. haemaphysaloides* can vector this pathogen remains to be determined. This work also reveals a high diversity of tick-borne bacteria and protozoans circulating in a small region and calls for further research on their potential risks for human health.

Keywords *Anaplasma phagocytophilum* · *Rickettsia* · Apicomplexa · Ticks · Tick-borne diseases · Taiwan

Introduction

Anaplasma phagocytophilum, the etiological agent of potentially fatal granulocytic anaplasmosis in humans and tick-borne fever in ruminants is transmitted mainly by hard ticks, particularly the *Ixodes* species (Woldehiwet 2000; Stuen et al. 2013). This pathogen can

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be transmitted transstadially but not transovarially in ticks; vertebrate reservoir hosts are necessary for infecting larval ticks to maintain *A. phagocytophilum* circulation in natural settings (Stuen et al. 2013).

Anaplasma phagocytophilum is widespread around the globe and is emerging in several places, including East Asia (Stuen et al. 2013). Human cases of granulocytic anaplasmosis have been confirmed with molecular methods in China (Zhang et al. 2008), Japan (Ohashi et al. 2013), and Korea (Kim et al. 2014), but not in Taiwan. However, that fact that *A. phagocytophilum* has been detected from rodents in Taiwan (Masuzawa et al. 2014) suggests that this pathogen also circulates in Taiwan and local people are at risks of getting infected with the disease. Previous study on *Anaplasma* infection in Taiwan has identified *A. platys* in hard ticks (*Rhipicephalus sanguineus*) collected from dogs (Yuasa et al. 2017), but detection of *A. phagocytophilum* has never been attempted in ticks collected from small mammals. This is despite the fact that *A. phagocytophilum* was found on Taiwanese rodents (Masuzawa et al. 2014) and ticks can vector this pathogen among rodent hosts and likely also to humans. One purpose of this study was thus to investigate *A. phagocytophilum* infection in ticks collected from small mammals. In addition, although infection of *Rickettsia* spp., the causative agent of human spotted fever, is widespread in ticks collected from shrews and rodents in Taiwan (Kuo et al. 2015a), the study focuses on geographical variation across Taiwan (Kuo et al. 2015a), leaving specific regions of Taiwan less intensively studied. One study that focuses on *Rickettsia* infection in ticks in eastern Taiwan (Hualien) reported only prevalence of infection but not the identity of the *Rickettsia* species (Kuo et al. 2012). Likewise, protozoans in hard ticks have rarely been investigated intensively in Taiwan although some disease-causing species, such as *Babesia microti* that causes babesiosis in humans, are also detected in rodents in Taiwan (Saito-Ito et al. 2008; Chao et al. 2017). Therefore, the other aim of this study is to intensively investigate *Rickettsia* and protozoan infection in ticks in Hualien, where ticks are common on shrews and rodents (Kuo et al. 2011a).

Materials and methods

Study area and tick collection

Ticks were collected during a surveillance project of ectoparasites on small mammals in Taiwan (Kuo et al. 2011a, b, 2012). Briefly, small mammal traps were set up in Ji-an, Shou-feng and Fong-lin townships in Hualien County, eastern Taiwan, during 2007 and 2008. Ticks were removed from trapped shrews and rodents, preserved in 70% ethanol, and stored at -70°C for later pathogen assay. Species of ticks were identified following published keys (Teng and Jiang 1991) and confirmed by comparing 12S rDNA sequences with known species (Beati and Keirans 2001).

Pathogen detection in ticks

Each tick was placed in a 2 ml eppendorf tube and crushed by shaking with steel grinding balls in a bead beater (TissueLyser II, Qiagen) for 2.5 min at a frequency of 30 oscillations per second, then individually assayed for infection of bacteria (*Anaplasma*, *Rickettsia*), and protozoans (Apicomplexa). Primers and methods for amplifying fragments of these organisms were provided in Table 1. Positive samples were sequenced twice in each direction

Table 1 Primers for tick-borne pathogen detections in hard ticks (Ixodidae) in Hualien, eastern Taiwan

	Gene target	Primers	Sequences (5'–3')	Product size (bp)	Method	Reference
<i>Anaplasma</i> spp.	16S rRNA gene	EHR 16SD EHR 16SR	GGTACC(C/T)ACAGAAGAAGTCC TAGCACTCATCGTTTACAGC	306	Real time PCR	Parola et al. (2000)
<i>Rickettsia</i> spp.	OmpB	rompB OF	GTAACCGGAAGTAATCGTTTCGTAA	426 or 250	Nested PCR	Choi et al. (2005)
		rompB OR	GCITTATAACCAAGCTAAACCAACC			
		rompB SFG IF	GTTTAATACGTGCTGCTAACCAA			
		SFG/TG IR	GGTTTGCCCATATACCATAAG			
		rompB TG IF	AAGATCCTTCTGATGTTGCAACA			
Apicomplexa spp.	gltA	RpCS.877p	GGGGGCTGCTCACGGCGG	338	Nested PCR	
		RpCS.1258n	AATGCAAAAAGTACAGTGAACA			
		RpCS.896	GGCTAATGAAGCAGTGATAA			
		RpCS.1233n	GCGACGGTATACCCATAGC			
	18S rRNA gene	BmF1	GCGATGTATCATTCAAAGTTTCTG	700	Nested PCR	Simpson et al. (2005)
		BmR1	TGTTATTGCCTTACACTTCCTTGC			
		BmF2	ACGGCTACCAATCTAAGGAAGGC			
		BmR2	TCTCTCAAGGTGCTGAAGGA			

and potential parasitic species were identified by resemblance to known species using the Basic Local Alignment Search Tool.

Results

Tick collection

A total of 270 *Rhipicephalus haemaphysaloides* ticks were assayed individually, including 155 larvae and 115 nymphs (no adult *R. haemaphysaloides* were collected). These ticks were collected from eight small mammal species, including two shrew (*Crocidura attenuate*, *Suncus murinus*) and six rodent species (*Apodemus agrarius*, *Bandicota indica*, *Mus caroli*, *M. musculus*, *Rattus exulans*, *R. losea*) (Table 2).

Pathogen assay

Two *Anaplasma* spp. were identified, including one *A. phagocytophilum* (100% identity to *A. phagocytophilum*, GenBank accession number: MG519284.1) in a nymphal *R. haemaphysaloides* collected from *A. agrarius* (prevalence 0.4%) in Shou-feng and 19 detections of bacteria most similar to *Anaplasma bovis* in 6 larvae and 13 nymphs (prevalence 7.0%) collected from *A. agrarius* and *R. losea* (Table 2). Likewise, two *Rickettsia* spp. were recognized, including 16 detections of *R. rickettsia*-like bacteria in 7 larvae and 9 nymphs (prevalence 5.9%) from *A. agrarius*, *B. indica*, and *R. losea*, and 74 detections of *Rickettsia* sp. TwKM01-like bacteria in 37 larvae and 37 nymphs (prevalence 27.4%) collected from all rodent species except for *M. musculus* (Table 2). Lastly, at least seven Apicomplexa species were identified, including nucleotide sequences most similar to two *Cryptosporidium* spp. (*C. galli*, *C. struthionis*), four *Hepatozoon* spp. (*Hepatozoon* sp. DB2231, *Hepatozoon* sp. HepBiCM001, *Hepatozoon* sp. pty01po, *Hepatozoon* sp. YLW-2014 isolate), and two detections of *Theileria* genus with unknown species. These Apicomplexa species were mostly identified once (prevalence 0.4%), except for *Hepatozoon* sp. DB2231 that were detected twice (prevalence 0.7%) and *Hepatozoon* sp. YLW-2014 isolate that were detected five times (prevalence 1.9%). These 13 Apicomplexa detections occurred to two larvae and 11 nymphs removed from *A. agrarius*, *M. caroli*, and *R. losea* (Table 2).

Discussion

In this study, *A. phagocytophilum* was identified in a hard tick for the first time in Taiwan. In addition, another *Anaplasma* sp. (*A. bovis*) also circulates locally. Lastly, we detected a moderate prevalence of *Rickettsia* infection and several Apicomplexa protozoan species. These results demonstrate a variety of tick-borne bacteria and protozoans circulating in eastern Taiwan and could pose health risks to local people.

We detected *A. phagocytophilum* in a *R. haemaphysaloides* tick collected from *A. agrarius* rodent, showing that *A. phagocytophilum* not only occurs in central Taiwan and Kinmen Island (Masuzawa et al. 2014) but also in eastern Taiwan. Furthermore, the pathogen was detected in *R. haemaphysaloides* that also bites humans (Guglielmone et al. 2014). It should be noted, nevertheless, that the detection of *A. phagocytophilum* doesn't mean that the tick can certainly vector the pathogen because the assayed tick was retrieved from the

Table 2 Number of assayed larval and nymphal *Rhipicephalus haemaphysaloides* ticks collected from small mammal hosts and identified bacteria (*Anaplasma* and *Rickettsia*) and Apicomplexa protozoan species in Hualien, eastern Taiwan

Host species	Number of ticks assayed		<i>Anaplasma</i> spp.	<i>Rickettsia</i> spp.	Apicomplexa spp.	
	Larva	Nymph				Total
Shrew						
<i>Crocidura attenuate</i>	1	0	1 ^a	–	–	
<i>Suncus murinus</i>	4	0	4	–	–	
Rodent						
<i>Apodemus agrarius</i>	86	56	142	<i>A. phagocytophilum</i> (1N); <i>A. bovis</i> (3L, 7N) ^b	<i>R. rickettsii</i> (2N); <i>Rickettsia</i> sp. TwKM01 (22L, 18N)	<i>Cryptosporidium galli</i> (1N); <i>Cryptosporidium struthionis</i> (1N); <i>Theileria</i> sp. (2N)
<i>Bandicota indica</i>	7	11	18	–	<i>R. rickettsii</i> (1N); <i>Rickettsia</i> sp. TwKM01 (1L, 5N)	–
<i>Mus caroli</i>	6	2	8	–	<i>Rickettsia</i> sp. TwKM01 (3L)	<i>Hepatozoon</i> sp. YLW-2014 (1L)
<i>Mus musculus</i>	1	1	2	–	–	–
<i>Rattus exulans</i>	10	3	13	–	<i>Rickettsia</i> sp. TwKM01 (4L, 2N)	–
<i>Rattus losea</i>	40	42	82	<i>A. bovis</i> (3L, 6N)	<i>R. rickettsii</i> (7L, 6N); <i>Rickettsia</i> sp. TwKM01 (7L, 12N)	<i>Hepatozoon</i> sp. DB2231 (2N); <i>Hepatozoon</i> sp. HepBiCM001 (1N); <i>Hepatozoon</i> sp. pty01po (1N); <i>Hepatozoon</i> sp. YLW-2014 (1L, 3N)
Total	155	115	270	<i>A. phagocytophilum</i> (1N); <i>A. bovis</i> (6L, 13N)	<i>R. rickettsii</i> (7L, 9N); <i>Rickettsia</i> sp. TwKM01 (37L, 37N)	<i>Cryptosporidium galli</i> (1N); <i>Cryptosporidium struthionis</i> (1N); <i>Hepatozoon</i> sp. DB2231 (2N); <i>Hepatozoon</i> sp. HepBiCM001 (1N); <i>Hepatozoon</i> sp. pty01po (1N); <i>Hepatozoon</i> sp. YLW-2014 (2L, 3N); <i>Theileria</i> sp. (2N)

^aNegative results^bNumber of larval (L) and nymphal (N) ticks detected

rodent host and *A. phagocytophilum* might come from un-digested blood meal, particularly when *A. agrarius* rodent is implicated as a main reservoir of *A. phagocytophilum* in other countries, such as China (Cao et al. 2006) and Korea (Kim et al. 2006). However, because *A. phagocytophilum* is transmitted mainly by ticks (Stuenkel et al. 2013) and the local tick fauna is dominated by *R. haemaphysaloides* (>99%, Kuo et al. 2011a), *R. haemaphysaloides* is the most likely candidate vector for propagating *A. phagocytophilum* transmission in our study area.

Likewise, *A. bovis* has been detected in small mammals in central Taiwan and Kinmen Island before (Masuzawa et al. 2014) and similar bacteria were detected in the hard ticks for the first time in Taiwan. This study and Masuzawa et al. (2014) both revealed the existence and only the existence of *A. phagocytophilum* and *A. bovis* in small mammals and associated ticks in Taiwan; besides, the latter bacteria was more common than the former one. *A. bovis* has been identified in ruminants and several other mammals groups around the world (Ben Said et al. 2015), including China (Liu et al. 2012), Japan (Yoshimoto et al. 2010), and Korea (Doan et al. 2013), so it might not be surprising that this pathogen also occurs in Taiwan. Likewise, the 19 detections of *A. bovis* came from *R. haemaphysaloides* ticks, which might be the local vector of *A. bovis*.

Rickettsia spp. commonly occur in small mammals (Kuo et al. 2015b) and associated ticks (Kuo et al. 2015a) across Taiwan. In this study, we found that *Rickettsia* sp. TwKM01-like bacteria was common in *R. haemaphysaloides* ticks in Hualien (prevalence 27.4%). Previous studies on *Rickettsia* infection in the same region have detected *Rickettsia* sp. TwKM01 in small mammals (Kuo et al. 2015b) but not in ticks (Kuo et al. 2015a), likely due to the small number of ticks (n=10) being assayed in Kuo et al. (2015a). *Rickettsia* sp. TwKM01 was first recognized in *R. haemaphysaloides* in Hualien and Kinmen Island and its pathogenicity remains to be determined (Tsui et al. 2007), but together these studies show that *Rickettsia* sp. TwKM01 was identified mostly in *R. haemaphysaloides* although it has also been detected in *Ixodes granulatus* ticks, one time each in Kinmen Island and Penghu Island (Kuo et al. 2015a).

Babesia microti that can cause babesiosis in humans was not found in this study; instead, we identified ≥ 7 protozoan species, including those in *Cryptosporidium*, *Hepatozoon*, and *Theileria*. Worldwide, these three genera include some species of medical and veterinary importance, such as *Cryptosporidium hominis* in humans, *Hepatozoon canis* in canids, and *Theileria annulata* in the livestock (Bouzid et al. 2013; Sumrandee et al. 2015). Pathogenicity of the ≥ 7 apicomplexan species recognized herein is unknown, but the many species identified in a small region calls for a more intensive and extensive study on this neglected group of parasites in Taiwan.

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