Tropical Biomedicine 37(3): 803–811 (2020)

Culturable pathogenic bacteria in ticks parasitizing farm animals and rodents in Malaysia

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Received 16 August 2019; received in revised form 16 December 2019; accepted 16 December 2019

Abstract. Ticks are vectors of bacteria, protozoa and viruses capable of causing serious and life threatening diseases in humans and animals. Disease transmission occurs through the transfer of pathogen from tick bites to susceptible humans or animals. Most commonly known tick-borne pathogens are obligate intracellular microorganisms but little is known on the prevalence of culturable pathogenic bacteria from ticks capable of growth on artificial nutrient media. One hundred and forty seven ticks originating from dairy cattle, goats and rodents were collected from nine selected sites in Peninsular Malaysia. The culture of surface-sterilized tick homogenates revealed the isolation of various pathogenic bacteria including, *Staphylococcus* sp., *Corynebacterium* sp., *Rothia* sp., *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Bacillus* sp. and its derived genera. These pathogens are among those that affect humans and animals. Findings from this study suggest that in addition to the regular intracellular pathogens, ticks could also harbor extracellular pathogenic bacteria. Further studies, hence, would be needed to determine if these extracellular pathogens could contribute to human or animal infection.

INTRODUCTION

Ticks are blood feeding ectoparasites of numerous hosts, including farm animals such as cattle and goats, rodents, birds and reptiles (Liu & Bonnet, 2014). They are also vectors of bacteria, protozoa and viruses capable of causing serious and life threatening diseases in humans and animals (Khoo *et al.*, 2016; Bell-Sakyi *et al.*, 2018). Amongst the bacterial species found within ticks, those in the genera of *Rickettsia*, *Anaplasma*, *Coxiella* and *Ehrlichia* that are commonly associated with human and animal infections (Khoo *et al.*, 2016) are obligate intracellular pathogens incapable of growing on artificial nutrient culture. As disease transmission from ticks to humans occur through tick bites (Loong *et al.*, 2018a), there is also risk of transmission of other pathogens carried within the ticks. To date, little is known on the prevalence of culturable pathogenic bacteria harbored within ticks. Bacterial culture and isolation are essential as they have long been accepted as the gold standard for laboratory confirmation of bacterial infections (Loong *et al.*, 2016).

The recent economic trade expansion, surging human travel, ecohabitat changes and the increasing geographical distribution

of several tick species have contributed to the emergence of tick-borne diseases (Liu & Bonnet, 2014). Trading and importation of farm animals provide a conduit for the entry of pathogen-carrying ticks into the respective countries. Between 2012 and 2018, Malaysia continuously experienced a supply shortage of fresh milk and mutton (Department of Veterinary Services, 2018). To meet local demand for these livestock products, the government has encouraged import from other countries (Shahudin *et al.*, 2018; Loong *et al.*, 2019). As a result, the importation of non-native farm animals could inadvertently introduce potentially exotic diseases in addition to the common tick-borne pathogens into Malaysia's ecosystem. The coexistence of rodents in close proximity to animal and human habitations potentially promote the transmission of zoonotic pathogens, in particular tick-borne pathogens. Rodents play an important role in harboring and spreading (via ticks) of numerous tick-borne pathogens including *Babesia microti*, *Borrelia burgdorferi*, *Rickettsia australis* and *Rickettsia rickettsii* (Meerburg *et al.*, 2009). Here, we present a baseline study of culturable bacterial pathogens of ticks recovered from dairy cattle, goat and rodent, collected from nine selected sites in Peninsular Malaysia.

MATERIALS AND METHODS

Parasitizing ticks were scoured from two types of farm animals (dairy cattle and goat) and rodents at multiple locations in Malaysia. The sampling of cow ticks was performed in three dairy cattle farms in Rantau and Mantin in Negeri Sembilan, and Banting in Selangor (Figure 1, Table 1). Ticks infesting on goats were collected from two farms in Teluk Intan and Bagan Datoh in Perak (Figure 1, Table 1). Meanwhile, ticks were collected off trapped rodents in the following sites in Selangor; Kampung Sungai Semungkis, Sungai Congkak, Ulu Perdik and Ulu Yam (Figure 1, Table 1). All ticks were stored in 70% ethanol prior to transportation to the laboratory for analyses. In the laboratory, collected ticks were surface sterilized with 70% ethanol and rinsed thoroughly with nuclease-free water to remove all possible environmental contaminants (Loong *et al.*, 2018a). Adult ticks were then sorted according to species, host, sex and blood meal status into pools of between 1 and

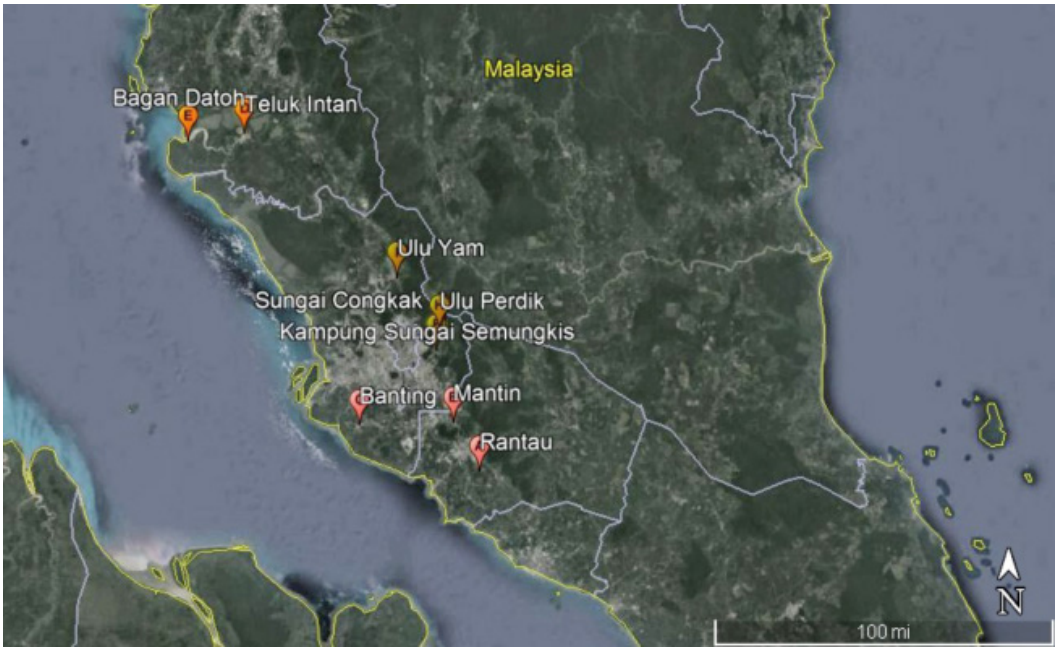

Figure 1. Sampling sites for the collection of ticks from farm animals and rodents.

Table 1. Geographical coordinates of sampling sites in this study

Sampling site	Latitude	Longtitude	No. of tick (n)	Animal host
Rantau, Negeri Sembilan	2.6200°N	102.0033°E	6	Cow
Mantin, Negeri Sembilan	2.8230°N	101.8942°E	18	
Banting, Selangor	2.8121°N	101.5026°E	14	
Teluk Intan, Perak	4.0224°N	101.0206°E	25	Goat
Bagan Datoh, Perak	3.9919°N	100.7861°E	14	
Kampung Sungai Semungkis, Selangor	3.1258°N	101.8232°E	21	Rodent
Sungai Congkak, Selangor	3.2087°N	101.8435°E	33	
Ulu Perdik, Selangor	3.2052°N	101.8359°E	4	
Ulu Yam, Selangor	3.4254°N	101.6596°E	12	

Table 2. Blood meal status and sex of ticks collected from the different animal hosts in this study

	Dairy cattle (n=38)		Goat (n=39)		Rodent (n=70)	
	F	M	F	M	F	M
Engorged (n=137)	29	9	29	0	69	1
Not engorged (n=10)	0	0	5	5	0	0

F, Female; M, Male.

5 individuals, before pulverization in liquid nitrogen using chilled mortar and pestle (Khoo *et al*., 2016). The resulting homogenates were cultured onto Columbia agar with 5% sheep blood and incubated aerobically at 37°C for 48 h. Bacterial cultures were streak-plated until single colonies were obtained and 16S rDNA sequencing was performed on the colonies to facilitate identification of the pathogen (Loong *et al*., 2016).

RESULTS

A total of 147 ticks were collected from dairy cattle, goats and rodents from nine selected locations in Peninsular Malaysia (Table 2). Amongst them, 132 were females, 15 were males and five of each were not engorged while 137 were engorged (Table 2). The majority of ticks were collected from rodents (n=70), followed by goats (n=39) and cattle (n=38) (Table 2). Sungai Congkak represented the location with the highest tick collection (n=33), whereas Ulu Perdik was the location with the least collected tick (n=4), both from rodent hosts (Table 1). The tick species which were determined using established morphological keys (Khoo *et al*., 2016), showed an almost homogenous disassociation between different hosts. *Rhipicephalus microplus* was found exclusively infesting dairy cattle, *Haemaphysalis bispinosa* solely on goats and, the majority of rodents with *Ixodes granulatus* and only one *Dermacentor* sp. was found on a *Maxomys surifer* rodent. The collected *Dermacentor* sp. could not be classified to the species level due to damaged gnathostome, crucial for tick species identification. *I. granulatus* was collected from six different rodent hosts (*M. surifer*, *Maxomys whiteheadi*, *Sundamys muelleri*, *Rattus rattus*, *Rattus tiomanicus* and *Leopoldamys sabanus*).

There were considerable genus and species diversity in the bacterial isolates cultured from the ticks. Bacteria from the genera *Bacillus*, *Corynebacterium*, *Rothia* and *Staphylococcus* were found in ticks collected from dairy cattle, with *Staphylococcus chromogenes* and *Staphylococcus sciuri* identified to the

species level (Table 3). Meanwhile, ticks collected from goats yielded *Bacillus* sp., *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Lysinibacillus* sp. (Table 4). The rodent ticks however, harbored bacteria closely related to the *Bacillus* genera. Amongst those cultured were *Bacillus* sp., *Brevibacillus* sp., *Lysinibacillus* sp. and *Paenibacillus lautus* (Table 5). Differences in the number of bacterial isolates were noted in the individual tick pools, some with up to three different isolated bacteria and some showing no bacterial growth. All specimens showing no bacterial growth were from pools of engorged females collected from cattle (2/45 pools) and rodents (18/45 pools), whereas pools of males (4/45 pools), irrespective of the blood meal status, spawned various bacterial isolates (Tables 3–5).

Table 3. Cultivable bacteria in ticks parasitizing dairy cattle

Pool # (individuals/ pool, n)	Location	Tick species	Tick sex	Blood meal status	Bacteria identity
1 (n=3)	RT	*R. microplus*	F	E	• *Staplylococcus* sp. • *Corynebacterium* sp. • *Bacillus* sp.
2 (n=3)	RT	*R. microplus*	F	E	• *Staplylococcus* sp.
3 (n=3)	MT	*R. microplus*	F	E	NIL
4 (n=3)	MT	*R. microplus*	F	E	NIL
5 (n=4)	MT	*R. microplus*	M	E	• *Staphylococcus* sp.
6 (n=4)	MT	*R. microplus*	F	E	• *Staphylococcus chromogenes*
7 (n=4)	MT	*R. microplus*	F	E	• *Staphylococcus* sp.
8 (n=5)	BT	*R. microplus*	F	E	• *Staphylococcus sciuri*
9 (n=4)	BT	*R. microplus*	F	E	• *Staphylococcus* sp. • *Corynebacterium* sp.
10 (n=5)	BT	*R. microplus*	M	E	• *Rothia* sp. • *Corynebacterium* sp.

RT, Rantau; MT, Mantin; BT, Banting; F, Female; M, Male; E, Engorged; NIL, No bacterial growth.

Table 4. Cultivable bacteria in ticks parasitizing goats

Pool # (individuals/ pool, n)	Location	Tick species	Tick sex	Blood meal status	Bacteria identity
1 (n=5)	TI	*H. bispinosa*	F	E	• *Enterococcus faecalis* • *Klebsiella pneumoniae* • *Bacillus* sp.
2 (n=5)	TI	*H. bispinosa*	F	E	• *Enterococcus faecalis* • *Klebsiella pneumoniae* • *Escherichia coli*
3 (n=5)	TI	*H. bispinosa*	F	X	• *Escherichia coli*
4 (n=5)	TI	*H. bispinosa*	F	E	• *Bacillus* sp. • *Lysinibacillus* sp.
5 (n=5)	TI	*H. bispinosa*	M	X	• *Bacillus* sp.
6 (n=5)	BD	*H. bispinosa*	F	E	• *Bacillus* sp.
7 (n=5)	BD	*H. bispinosa*	F	E	• *Bacillus* sp.
8 (n=4)	BD	*H. bispinosa*	F	E	• *Bacillus* sp.

TI, Teluk Intan; BD, Bagan Datoh; F, Female; M, Male; E, Engorged; X, Not engorged.

Table 5. Cultivable bacteria in ticks parasitizing rodents

Pool # (individuals/ pool, n)	Location	Rodent host	Tick species	Tick sex	Blood meal status	Bacteria identity
1 (n=1)	SC	*M. surifer*	*Dermacentor* sp.	F	E	NIL
2 (n=1)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp. • *Brevibacillus* sp. • *Lysinibacillus* sp.
3 (n=1)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp.
4 (n=1)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp. • *Brevibacillus* sp.
5 (n=1)	SC	*S. muelleri*	*I. granulatus*	M	E	• *Paenibacillus lautus* • *Bacillus* sp. • *Brevibacillus* sp.
6 (n=1)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp.
7 (n=1)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp.
8 (n=3)	SC	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp.
9 (n=3)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
10 (n=3)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
11 (n=2)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
12 (n=2)	SC	*R. rattus*	*I. granulatus*	F	E	NIL
13 (n=1)	SC	*M. surifer*	*I. granulatus*	F	E	NIL
14 (n=4)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
15 (n=5)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
16 (n=3)	SC	*S. muelleri*	*I. granulatus*	F	E	NIL
17 (n=4)	KSS	*S. muelleri*	*I. granulatus*	F	E	NIL
18 (n=3)	KSS	*S. muelleri*	*I. granulatus*	F	E	NIL
19 (n=3)	KSS	*S. muelleri*	*I. granulatus*	F	E	NIL
20 (n=3)	KSS	*S. muelleri*	*I. granulatus*	F	E	• *Bacillus* sp.
21 (n=2)	KSS	*M. whiteheadi*	*I. granulatus*	F	E	NIL
22 (n=3)	KSS	*S. muelleri*	*I. granulatus*	F	E	• *Lysinibacillus* sp.
23 (n=3)	KSS	*L. sabanus*	*I. granulatus*	F	E	NIL
24 (n=4)	UP	*R. tiomanicus*	*I. granulatus*	F	E	NIL
25 (n=4)	UY	*S. muelleri*	*I. granulatus*	F	E	NIL
26 (n=4)	UY	*S. muelleri*	*I. granulatus*	F	E	NIL
27 (n=4)	UY	*S. muelleri*	*I. granulatus*	F	E	NIL

SC, Sungai Congkak; KSS, Kampung Sungai Semungkis; UP, Ulu Perdik; UY, Ulu Yam; F, Female; M, Male; E, Engorged; NIL, No bacterial growth.

DISCUSSION

We employed a non-selective bacterial culture method in this study, previously shown to enable isolation of rare and pathogenic bacteria species from tick specimens such as *Corynebacterium lactis* (Lim *et al*., 2018) and *P. lautus* (Loong *et al*., 2018a). Even though no bacterial growth was observed for some tick specimens, we suggest that it was not due to the limitation of the agar culture media but the method employed for bacterial isolation (Stewart, 2012). Tick cell lines (Bell-Sakyi *et al*., 2018) could perhaps be useful for the cultivation of non-culturable bacterial pathogens. The simultaneous isolation of up to three different bacterial species in the collected tick specimens was not unexpected, since previous microbiome studies performed based on next generation sequencing technologies have identified up to hundreds of bacterial genera associated to a single or pooled tick specimens (Khoo *et al*., 2016; Trout Fryxell *et al*., 2016; Estrada-Peña *et al*., 2018; Lado *et al*., 2018). These microbiome

studies have identified numerous tick-borne pathogens (i.e. *Rickettsia* and *Anaplasma*) as well as other bacteria which were endogenous to the tick itself, or may have originated from the environment or the animal skin microbiota or the host blood meal (Moreno *et al*., 2006; Van Treuren *et al*., 2015; Clow *et al*., 2018; Estrada-Peña *et al*., 2018). Despite alcohol washing, some of the bacteria may have survived, perhaps in crevices of the exoskeleton not reachable by the alcohol. Bacteria from the genera *Staphylococcus*, *Bacillus*, *Corynebacterium*, *Klebsiella* and *Rothia*, some of which isolated in this study, were reported across many tick species (Andreotti *et al*., 2011; Carpi *et al*., 2011; Tveten *et al*., 2013; Clow *et al*., 2018; Ruiling *et al*., 2019), suggesting that these bacteria may have a role in the biology of the tick or the transmission of tick pathogens. *Staphylococcus* and *Corynebacterium* were detected in the gut tissues of ticks in previous studies, suggesting that ticks may be vectors of these bacteria (Abraham *et al*., 2017). However, since these bacteria were not normally considered as tick pathogens, they were rarely studied. This study represents an attempt at culturing these tick-associated bacteria, which have only been identified molecularly from ticks in past studies.

Staphylococcal infections among farm animals have been intensively studied, particularly on methicillin-resistant *S. aureus* and other species that cause mastitis (Foster, 2012). Although we were unsurprised by the finding of *Staphylococcus* sp. in *R. microplus* ticks found on dairy cattle, we were confounded by their absence in goat ticks. Since most *Staphylococcus* sp. are innocuous commensals on livestock skin (Foster, 2012), one explanation could be that the culture methods used in this study favored pathogenic strains, evolutionary-primed for survival over commensal species (Loong *et al*., 2018b). This was illustrated by the isolation of *Corynebacterium* sp., *S. chromogenes* and *S. sciuri*, all pathogenic bovine mastitis causing bacteria (Gonçalves *et al*., 2016; Khazandi *et al*., 2018), from *R. microplus* tick pools. *Rothia* sp. is another bacteria capable of causing tissue infection in young, immunocompetent human (Tomczak *et al*., 2013) found in one male cow tick pool. In Malaysia, the majority of goat farms are still poorly managed leading to lamentable hygiene conditions usually with swarms of flies (Shahudin *et al*., 2018; Lim *et al*., 2019). These flies that are drawn to the faeces can then transmit the goat's gut bacteria to other areas (Ranjbar *et al*., 2016). This could explain the discovery of *E. coli*, *E. faecalis* and *K. pneumoniae*, all members of the goat gut microflora (Kim *et al*., 2017) and also potential human pathogens (Tian *et al*., 2018), in *H. bispinosa* ticks collected from the body of goats. *I. granulatus* ticks collected from rodents however, yielded only bacteria from the *Bacillus* and *Bacillus*-derived genera. *Bacillus* sp. and *Lysinibacillus* sp. were also found among ticks collected from dairy cattle and goats. This observation is in agreement with an earlier study that found bacteria from the genus *Bacillus* being the most dominant genus isolated from *Ixodes* ticks in the United States (Martin & Schmidtmann, 1998). The predominance of these bacterial genera (*Bacillus* and *Bacillus*-derived genera) to environmental niches suggests that the isolated bacteria presumably originated from the environment too (Mukhtar *et al*., 2018). This finding should not be downplayed regardless, as environment-associated *Bacillus* sp. appears to have pathogenic potential in humans (Loong *et al*., 2017).

In conclusion, findings of the current study convey the impression that in addition to common intracellular tick-borne pathogens such as the rickettsial agents, other extracellular bacterial pathogens associated with human or animal infections could also be recovered and this provide a baseline information of the culturable bacterial species in ticks of farm animals and rodents. This study represents an attempt in culturing these tick-associated extracellular bacteria, which provides the opportunity for future research in determining their roles in tick biology and the transmission of pathogens by ticks, also highlighting the importance of considering other potential pathogens carried within ticks.

Acknowledgements. We acknowledge the funding from the Ministry of Education, Malaysia for niche area research under the Higher Institution Centre of Excellence (HICoE) program (Project MO002-2019). This study was also supported in parts by research grants from the University of Malaya, Malaysia, under the Research University grant (RU008-2018) and the UMCoE Top 100 research grant (UM.00000188/HGA.GV).

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