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Isolation and phylogenetic analysis of *Bartonella* species from Rusa deer (*Rusa timorensis*) in Thailand

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ABSTRACT

The aim of the present study is to investigate the prevalence of *Bartonella* infection in deer in Thailand and to characterize the isolates by biochemical, morphological and genetic analysis. A total of 247 blood samples were collected from Rusa deer (*Rusa timorensis*) in a livestock breeding facility in Thailand. *Bartonella* bacteria were isolated in 3.6% of the blood samples. Three out of 110 (2.7%) males and 6 of 137 (4.4%) females were positive for *Bartonella*. A higher prevalence of *Bartonella* was observed in young deer under 4 years of age compared to adults over 4 years of age, but no *Bartonella* was isolated from deer over 8 years of age. Phylogenetic analysis of concatenated sequences of seven loci of *Bartonella* indicated that all the isolates from Rusa deer in Thailand were identical and formed a distinct cluster from other known *Bartonella* species.

1. Introduction

Bartonella are small, fastidious, Gram-negative aerobic bacilli that are mainly transmitted by arthropod vectors. These microorganisms infect erythrocytes of their mammalian hosts and some *Bartonella* species are the cause of a wide spectrum of human illnesses, such as cat-scratch disease, chronic bacteremia, fever and endocarditis [1,2].

B. schoenbuchensis was first isolated from wild roe deer (*Capreolus capreolus*) in Germany [3]. Another deer-associated *Bartonella* species, *B. capreoli,* was also isolated from roe deer in France [4] and later found in elk (*Cervus elaphaus*) in the USA [5] and Japanese sika deer (*Cervus nippon*) in Japan [6]. *B. bovis* was first detected in a cow in France [4] and then also found in elk and mule deer (*Odocoileus hemionus*) in the USA [7]. Recently, *Bartonella* infections have also been found in moose (*Alces alces*) in Finland and Norway, but the *Bartonella* species were not identified [8,9]. Thus, ruminants including wild deer seem to be a reservoir of several *Bartonella* species in Europe, the USA and Japan.

Bartonella bacteria in wild deer are strongly suspected to be transmitted by deer keds. In Germany, *B. schoenbuchensis* was successfully isolated from deer keds (*Lipoptena cervi*) collected from roe deer and red deer (*Cervus elaphus*) [10] and deer keds (*L. cervi*) and ticks (*Ixodes ricinus*) from red deer in the Netherlands [11]. The DNA of *B. schoenbuchensis* was also detected by PCR in deer keds collected from white-tailed deer (*Odocoileus virginianus*) in Massachusetts, USA [12] as well as from moose in Finland [8]. Moreover, *Bartonella* DNA was also found in the neotropical deer keds (*L. mazamae*) from white-tailed deer in Georgia and South Carolina, USA [13]. These data suggest that deer keds may play an important role in transmitting *Bartonella* among wild deer populations in Europe and the USA.

Several deer species such as Sambar deer (*Rusa unicolor*), Rusa deer (*Rusa timorensis*), Axix deer (*Axis axis*) and red deer now habit in many places in Thailand. However, there is no information regarding the prevalence of *Bartonella* infections and its genetic properties in these deer species in the country. The objectives of this study were to investigate the prevalence of *Bartonella* bacteria among Rusa deer in Thailand and to characterize the deer strains using bacteriological and genetic techniques.

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Table 1
Prevalence of *Bartonella* by gender and age of deer (*Rusa timorensis*) in Thailand.

Group (Age, year)	No. positive/No. examined (%)		
	Male	Female	Total
Young (0 < 4)	2/48 (4.2)	5/40 (12.5)	7/88 (8.0)*
Adult (4 ≤ 8)	1/48 (2.1)	1/93 (1.1)	2/141 (1.4)
Aged (8 <)	0/14	0/4	0/18
Total	3/110 (2.7)	6/137 (4.4)	9/247 (3.6)

* Prevalence in young deer was significantly higher than in adult ($p < 0.01$).

2. Materials and methods

2.1. Samples

A total of 247 blood samples were collected from Rusa deer between February and March 2011 at Nongkwang Livestock Breeding and Research Center in the Ratchaburi Province, Thailand. After capture, deer were treated with ivermectin to remove ectoparasites. The animals were divided into three groups according to age including: a young group under 4 years of age (n = 88); an adult group between 4 and 8 years of age (n = 141); and an aged group over 8 years of age (n = 18). Five ml of blood were aseptically collected from the jugular vein and transferred to EDTA-containing collection tubes. The samples were sent frozen to the National Institute of Health, Thailand and stored at −80 °C until examination.

2.2. Isolation of Bartonella

Frozen blood samples were thawed at room temperature and 200 µl were centrifuged at 1,800 × *g* for 70 min. After centrifugation, the supernatant was discarded and 100 µl of the sediment was mixed with the same volume of medium 199 supplemented with sodium pyruvate and fetal bovine serum (Life technologies, USA) [6]. The mixture was plated on heart infusion agar (HIA: Difco, USA) plates containing 5% defibrinated rabbit blood. The agar plates were incubated at 35 °C under 5% CO_2 for up to 2–4 weeks [14]. Gram negative coccobacilli growing in small, rough and greyish colonies that required long culture periods (1 week or more) were tentatively regarded as *Bartonella*. Two or three colonies were picked from each plate and sub-cultured for further characterization with the same conditions as the primary culture.

2.3. Biochemical analysis

Indole, indoxyl phosphate, nitrate, urease and trehalose hydrolysis activities were tested by conventional standard method with fresh cultures. Catalase activity was tested by mixing fresh cultures and 3% H_2O_2 on a glass slide and checking for the presence of microbubbles within one minute. Other bacterial enzyme activities were tested using the MicroScan® RAID Anaerobe Panel (SIEMENS, USA) according to the manufacturer's instructions.

2.4. Electron microscopy

For electron microscopic analysis, the strains were plated on cover slip (coated with Poly-L-Lysine) suspended with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated with ethanol series of 30%, 50%, 70%, 95% and 100%, dried with a critical point dryer (Leica, EM CPD300, Austria) and coated with platinum. Cell morphology and surface structures were analyzed with a field-emission-type scanning electron microscope (JEOL model JSM-7610F, Japan) at 2.0 kV.

2.5. PCR amplification and DNA sequencing of the rpoB, gltA, ftsZ, groEL, ribC, 16S rRNA genes and the ITS region

Genomic DNA was extracted from each isolate using InstaGene Matrix (Bio-Rad, Canada). The primers targeting the β-subunit of the RNA polymerase (*rpoB*) [15], citrate synthase (*gltA*) [16], cell division-associated protein (*ftsZ*) [17], heat-shock protein (*groEL*) [18],

Table 2
Phenotypic characteristics of strain Pangjai-1 and other five strains of *Bartonella* species.

Characteristic	Pangjai-1	*B. japonica**	*B. silvatica**	*B. bovis***	*B. capreoli***	*B. schoenbuchensis****
oxidase	nt	−	−	−	−	nt
catalase	−	−	−	−	−	nt
Indole	−	nt	nt	nt	nt	nt
indoxyl phosphate	−	nt	nt	nt	nt	nt
nitrate	−	nt	nt	nt	nt	nt
urease	−	−	−	−	−	nt
trehalose	−	−	−	+	+	nt
arylamidase	nt	+	+	+	nt	nt
leycine	+	+	+	+	+	+
methionine	+	+	+	+	+	nt
lysine (alkaline)	+	+	+	nt	nt	nt
lysine (acidic)	+	+	+	+	+	nt
glycylglycine	+	+	+	+	+	nt
glycine	+	+	+	+	+	+
proline	+	−	+	+	+	+
arginine	+	+	+	+	+	+
tryptophan	+	+	+	+	+	+
pyrrolidonyl	−	−	−	−	−	nt
bis-*p*-nitrophenyl phosphate	+	nt	+	+	+	−
p-nitrophenyl-*β*-D-galactopyranoside	−	nt	nt	nt	nt	nt
p-nitrophenyl-*α*-D-galactopyranoside	−	nt	nt	nt	nt	nt
p-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide	−	−	−	nt	nt	nt
p-nitrophenyl-*α*-D-glucopyranoside	−	nt	nt	nt	nt	nt
o-nitrophenyl-*β*-D-glucopyranoside	−	nt	nt	nt	nt	nt
disodium *p*-nitrophenylphosphate	−	nt	nt	nt	nt	nt
p-nitrophenyl-*α*-L-fucopyranoside	−	nt	nt	nt	nt	nt
p-nitrophenyl-*α*-D-mannopyranoside	−	nt	nt	nt	nt	nt
N-acetyl-*β*-D-glucosaminide	nt	nt	nt	+	+	nt

+:positive, −:negative, nt: not tested, *:Inoue et al., 2010, **:Bermond et al., 2002, ***:Dehio et al., 2001.