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Short Communication

Molecular detection of *Bartonella* spp. in Malaysian small flying foxes (*Pteropus hypomelanus*)

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Abstract. *Bartonella* spp. are emerging zoonotic pathogens responsible for a wide variety of clinical syndromes in humans. Bats have been increasingly reported as reservoirs for *Bartonella* spp. In this study, molecular investigation showed the presence of *Bartonella* DNA in two of 30 blood samples of Malaysian small flying foxes (*Pteropus hypomelanus*). Two strains (*Bartonella* sp. KS013a and KS013b) were isolated from a PCR-positive blood sample after five days of incubation on blood agar. Based on the dendrogram constructed from 16S rRNA gene sequences, the two strains were genetically most closely related to ruminant associated *Bartonella* spp. Both strains are regarded as potentially novel *Bartonella* species as their citrate synthase (*gltA*) sequences exhibit less than 96% similarities to all previously identified *Bartonella* spp. Additionally, high *gltA* sequence similarity was observed between the strains with that reported from a bat fly (*Cyclopodia horsfieldi*) collected from *P. hypomelanus*. Possible transmission of *Bartonella* infection through bat flies and the impact of the infection in *P. hypomelanus* are yet to be investigated.

INTRODUCTION

Bartonella spp. are small Gram-negative bacteria which have been recognised as emerging zoonotic pathogens responsible for a number of human diseases including Carrion's disease (*Bartonella bacilliformis*), trench fever (*Bartonella quintana*), and cat scratch fever (*Bartonella henselae*). The bacteria infect red blood cells of humans and animals and can be transmitted through haematophagous arthropods, such as fleas, lice, flies, and ticks (Breitschwerdt *et al.*, 2013). Reports are increasing on bats as asymptomatic reservoirs for *Bartonella* spp. across several continents, including Asia. Based on sequence analysis of the citrate synthase (*gltA*) gene, ten phylogroups of *Bartonella* spp. have been reported in the blood samples of bats (*Rhinolophus*, *Hipposideros*, *Megaderma* and *Megaerops* spp.) in Southern Vietnam (Anh *et al.*, 2015). Han *et al.* (2017) reported ten groups of *Bartonella* species in the insectivorous bats of Northern China, while McKee *et al.* (2017) reported seven genogroups of *Bartonella* spp. in Thai bats. *Bartonella* spp. have also been reported from bat flies i.e., *Cyclopodia horsfieldi*, *Phthiridium (Stylidia) fraternal*, and *Basilia (Tripselia) coronata* (Morse *et al.*, 2012).

The presence of *Bartonella* infection in *Pteropus hypomelanus* has not been documented. *P. hypomelanus* (also known as the variable flying fox) is predominantly found on small off-shore islands in the Indo-Australian region and has been suggested as a reservoir host for Nipah

virus (Wacharapluesadee *et al.*, 2016; Yob *et al.*, 2001). To learn more about *Bartonella* infection in *P. hypomelanus*, molecular methods were applied in this study for detection of *Bartonella* DNA in blood samples obtained from a *P. hypomelanus* colony caught in a small island in Peninsular Malaysia.

MATERIALS AND METHODS

Blood sampling

A total of 30 blood samples from *P. hypomelanus* were provided by the Malaysian Department of Wildlife and National Parks, a governmental organisation responsible for the management and preservation of wildlife in Peninsular Malaysia. Bat trapping was conducted using mist nets at Kampung Tekek, Pulau Tioman, Peninsular Malaysia (104°10'E, 2°47'N) during a routine population management programme in April, 2016. Bats were anaesthesized with Zoletil® 50 (Tiletamine-Zolezepam, Virbac) and blood sampling was conducted through the alar vein. Besides blood samples, swabs from other body sites were also collected but not provided for investigation in this study. Bats were released after the sampling procedures.

DNA extraction and PCR assays

Blood specimens were collected and kept in liquid nitrogen prior to DNA extraction using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). The DNA samples were screened for *Bartonella* spp. using polymerase chain reaction (PCR) assays targeting the 16S-23S rRNA intergenic region (Maggi *et al.*, 2005). The assays were performed in aliquots of 25 µL reaction mixture containing 0.2 µL of DreamTaq DNA polymerase (Thermo Scientific, USA), 2.5 µL of 10X DreamTaq Green Buffer (Thermo Scientific, USA), 0.5 µL of 10 mmol/L dNTP Mix (Thermo Scientific, USA), 19.6 µL of sterile distilled water, 2 µL of DNA template and 0.1 µL of each 25 µmol/L primer (321s forward: 5'- AGA TGA TGA TCC CAA GCC TTC TGG- 3' and 983as reverse: 5'- TGT TCT YAC AAC AAT GAT GAT G- 3'). The PCR reaction included a denaturation step at 95°C for 5 min followed by 45 cycles of denaturation at 94°C for 45s, annealing at 54°C for 45s, extension at 72°C for 45s and a final extension step at 72°C for 10 min in a Veriti thermal cycler (Applied Biosystems, USA). A sample was considered positive when an amplified DNA fragment was detected upon agarose gel electrophoresis. Sterile distilled water and DNA extract obtained from *Bartonella elizabethae* strain BeUM (Tay *et al.*, 2016) were used as negative and positive controls, respectively. The amplified fragments were sequenced in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA), using forward and reverse primers of the assay.

Culture and molecular analyses of *Bartonella* strains

Each PCR-positive blood sample (50 µL) was mixed with an equal volume of sterile phosphate-buffered saline and cultured on a commercially available fresh Columbia agar supplemented with 5% sheep blood (Isolabs Sendirian Berhad, Malaysia) at 37°C in 5% CO_2. Suspected colonies on blood agar were streaked on fresh agar plates prior to DNA extraction. Amplification of the 16S-23S rRNA intergenic region (Maggi *et al.*, 2005), 16S rRNA (Norman *et al.*, 1995; Weisburg *et al.*, 1991), and citrate synthase (*gltA*) genes (Norman *et al.*, 1995) was performed on the bacterial DNA extracts. Sequence determination of the amplified fragments was performed in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA), using forward and reverse primers of each PCR assay. The sequences obtained were searched for homologous sequences in the GenBank database by using the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Dendrograms were constructed based on the 16S rRNA and *gltA* gene sequences, using the neighbor-joining method (Jukes-Cantor) in MEGA software and bootstrap analysis with 1,000 resamplings (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION

Bartonella DNA was amplified from 2/30 *P. hypomelanus* blood samples (KS013 & KS017) using PCR screening assays targeting the 16S-23S rRNA intergenic region. The sequence obtained from one sample (KS017, 350 nt, GenBank accession no. KY677750) demonstrated the highest sequence similarity (82%, 283/347 bp) with an uncultured *Bartonella* sp. clone #281 (GenBank accession no. KX420731) reported in the kidney of a *Rhinolophus euryale* bat in Georgia (Bai *et al.*, 2017). The other amplified fragment (KS013) produced noisy sequence data with the observation of multiple peaks in the chromatogram and was not able to be analysed.

Of the two PCR-positive samples, only KS013 blood sample was available for culture. Upon culturing, two strains producing round, smooth and greyish colonies with different diameters were observed on blood agar after five days of incubation (herein referred as *Bartonella* sp. KS013a and KS013b for big and small colony variants, respectively). The 16S rRNA gene sequences (1348 bp) of *Bartonella* sp. KS013a (Genbank accession no. KY677754) and KS013b (Genbank accession no. KY677755) were almost identical, except for two nucleotides. The sequences show the highest sequence similarities (98.9 and 98.8%, respectively) with that of *Bartonella* sp. Pangjai-1 (GenBank accession no. JX006076, unpublished) reported in a deer (*Rusa timorensis*) in Thailand. The dendrogram constructed based on the 16S rRNA gene sequences (Figure 1) reveals the clustering of *Bartonella* sp. KS013a and KS013b strains with *Bartonella* sp. Pangjai-1 and ruminant-associated *Bartonella* spp., i.e., *Bartonella bovis*, *Bartonella schoenbuchensis* and *Bartonella chomelii*. The grouping of bartonellae from bat flies with ruminant-associated *Bartonella* spp. has also been reported by Morse *et al.* (2012). *B. bovis* has been reported to cause bacteremia and endocarditis in beef and dairy cattle (Rudoler *et al.*, 2014), while *B. schoenbuchensii*, initially isolated from the deer ked *Lipoptena cervi*, has been implicated as the etiology of deer ked dermatitis in humans (Dehio *et al.*, 2004). *Bartonella chomelii*, first isolated from a domestic cattle (Maillard *et al.*, 2004), is the most frequent species infecting cattle grazing in communal mountain pastures in Spain (Antequera-Gomez *et al.*, 2015).

As *Bartonella* 16S rRNA gene provide poor phylogenetic resolution due to its low sequence diversity (La Scola *et al.*, 2003), *gltA* was used to clarify the phylogenetic position of the bartonellae isolated in this study relative to those from other bats. BLAST analysis of the *gltA* sequences obtained from KS013a (Genbank accession no. KY677752) and KS013b (Genbank accession no. KY677753) revealed the highest sequence similarities (99.6%, 276/277 bp and 96.2%, 254/264 bp, respectively) with that of an uncultured bacterium clone E7 (Genbank accession no. JX416257) which was previously reported from an adult bat fly (*Cyclopodia horsfieldi*) in Malaysia (Morse *et al.*, 2012). As both strains in this study displayed only low sequence similarity (91.2 and 90.2%, respectively) with the closest validated species, *B. quintana* strain RM-11 (GenBank accession no. CP003784) (Figure 2), they are thus considered novel based on the species definition by La Scola *et al.* (2003), whereby an isolate should be regarded as a new species if the *gltA* sequence exhibits less than 96% similarity with a validated *Bartonella* species. New *Bartonella* species have also been reported in bats from Southern Vietnam, Thailand and Northen China. Of the ten phylogroups of *Bartonella* spp. reported in the blood samples of bats (*Rhinolophus*, *Hipposideros*, *Megaderma* and *Megaerops* spp.), nine are considered potentially novel due to low *gltA* sequence similarity (Anh *et al.*, 2015). Of the 10 phylogroups identified for *Bartonella* spp. in bats from Northern China, 5 strains appeared to be novel *Bartonella* species (Han *et al.*, 2017), while seven genogroups were found to be novel in the Thai bats (McKee *et al.*, 2017).

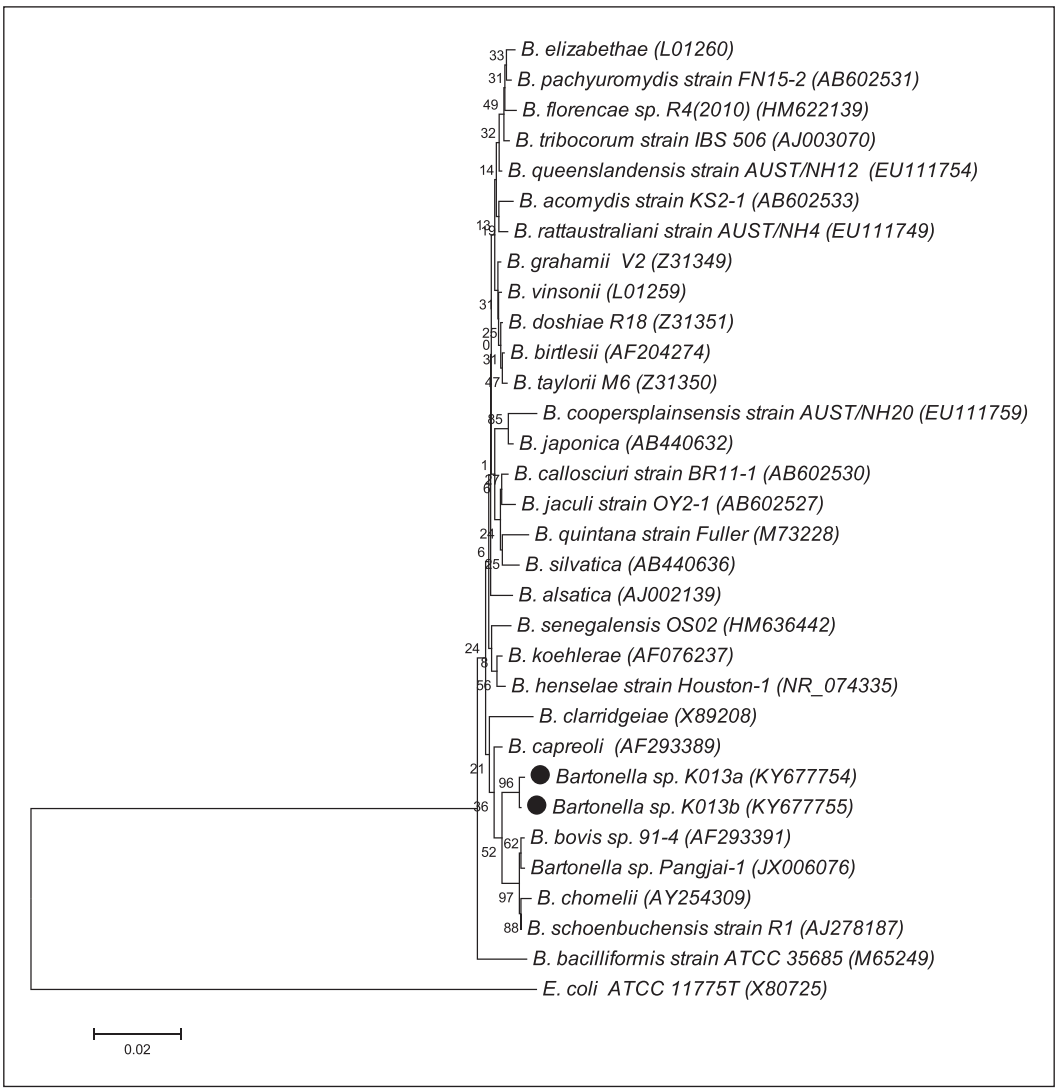

Figure 1. Dendrogram depicting the phylogenetic relationship of *Bartonella* sp. KS013a and KS013b (●) with *Bartonella* reference strains, based on the almost full length sequences of the 16S rRNA gene (1348 bp). *Escherichia coli* ATCC 11775T (X80725) was used as an outgroup. Numbers in brackets are GenBank accession numbers.

Figure 2 shows the dendrogram constructed based on the partial *gltA* sequences of KS013a and KS013b strains, a number of *Bartonell*a reference strains and six most similar sequences in the Genbank database (including three from bat blood samples, Genbank accession no. KX300117, KP100350, and KM030523; and three from bat flies, JX416255, JX416257 and JN172037). Both strains are clustered with clone E7 on a single branch (with a bootstrap value of 64%) and were close to *Bartonella* spp. from bat and bat flies, suggesting that they are likely members of a large clade of bat-associated *Bartonella* species as reported in recent studies (Morse *et al.*, 2012; Anh *et al.*, 2015; Bai *et al.*, 2015; Billeter *et al.*, 2012; Urushadze *et al.*, 2017). The high sequence similarity of both strains with the clone E7 (derived from a bat fly) also supports the notion on the possible role of bat fly as a vector or maintenance host for *Bartonella*

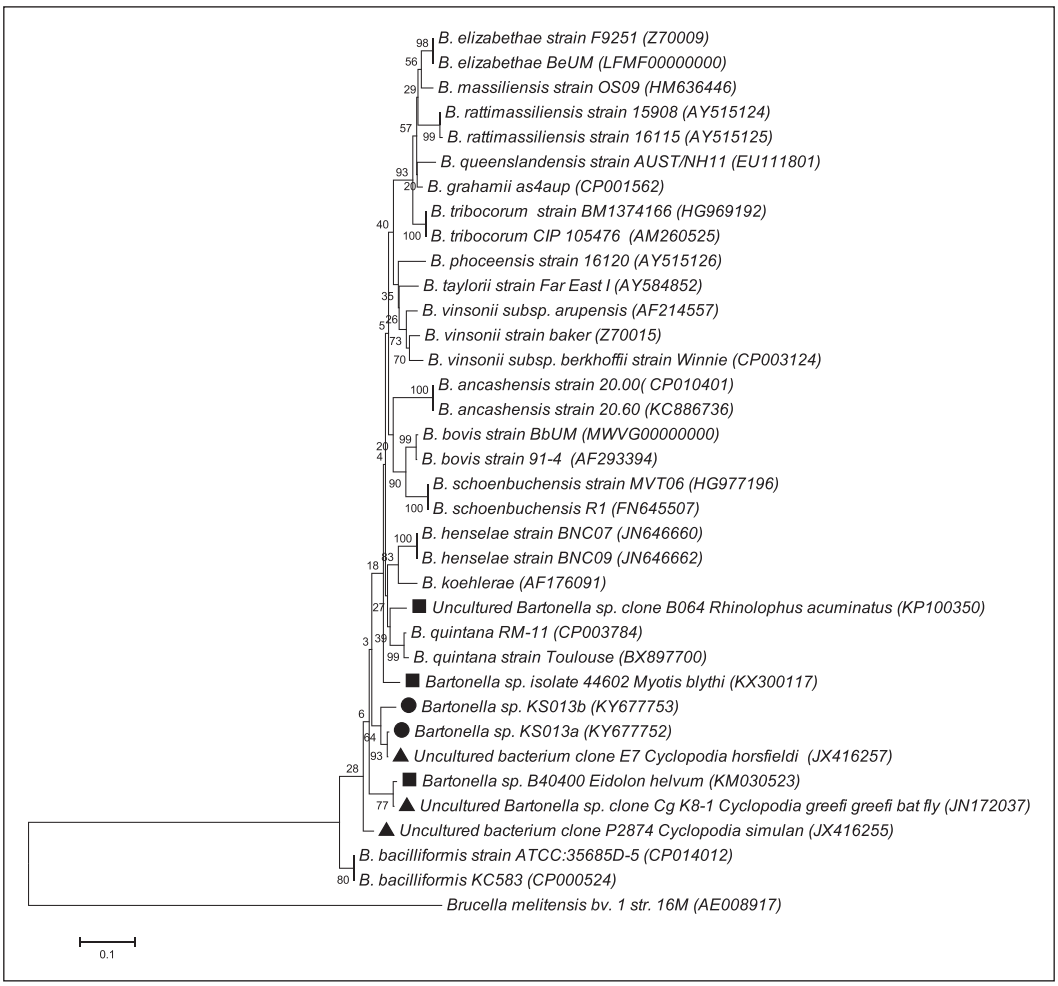

Figure 2. Dendrogram depicting the phylogenetic relationship of *Bartonella* sp. KS013a and KS013b (●) with *Bartonella* reference strains and six most similar sequences available in the Genbank database (including three from bat samples (■), KX300117, KP100350, and KM030523; and three from bat flies (▲), JX416255, JX416257 and JN172037) based on partial *gltA* sequences (263 bp). *Brucella melitensis* bv. 1 str. 16M (AE008917) was used as an outgroup. Numbers in brackets are GenBank accession numbers.

infection (Brook *et al.*, 2015; Wilkinson *et al.*, 2016). It is possible that *C. horsefieldi* is a vector of bartonellae for *P. hypomelanus*, as other *Pteropus* species (*P. lylei* and *P. vampyrus*) are also known to carry *C. horsefieldi* in Southeast Asia (Olival *et al.*, 2013).

Bats are highly mobile and have been frequently reported as the reservoirs for human pathogens. Most *Bartonella* spp. cause persistent bacteremia in their mammalian hosts. The pathogenic role of *Bartonella* spp. to *P. hypomelanus* is yet to be investigated. Further studies on the transmission dynamics of *Bartonella* infection and vector competency of bat flies are necessary. Additionally, genomic and microbiological characterization of *Bartonella* sp. KS013a and KS013b from *P. hypomelanus* will enhance our understanding on the host adaptability of *Bartonella* species.

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