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Original article

First detection and molecular identification of *Babesia microti* in *Rattus losea* captured from the offshore Kinmen Island of Taiwan

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ABSTRACT

Babesia microti was firstly detected and identified in brown country rats (*Rattus losea*, Swinhoe) captured from the offshore Kinmen Island of Taiwan. The prevalence of *Babesia* infection in 283 rodents was screened by polymerase chain reaction (PCR) assay using a piroplasma-conserved primer set (Piro A/B) and the thirty-seven PCR-positive rodents were further examined by PCR using a species-specific primer set (Bab 1/4) targeting the gene encoding the nuclear small-subunit ribosomal RNA (18S rRNA) of *Babesia* species. *B. microti* was detected only in *Rattus losea* with a total infection rate of 9.9% (28/283). Positivity examined by species-specific PCR (9.9%) is higher than examined by blood smear (4.6%). Sequence and phylogenetic analyses revealed that *Babesia* species detected in Taiwan were genetically affiliated to the genotypes of *B. microti*, and can be easily distinguished from other genotypes of *Babesia* parasites by neighbour-joining and maximum-parsimony methods. Intra- and inter-species analysis also indicate that all these Taiwan species have a lower level of genetic divergence (genetic distance values <0.084) within the genotypes of *B. microti*, and were genetically more distant to other genotypes (>0.218) of *Babesia* parasites. This study provides the first evidence of *B. microti* identified in *R. losea* in Taiwan, and the high prevalence of *Babesia* infection in *R. losea* may imply its possible role served as reservoir host for maintaining an enzoonotic cycle of *Babesia* transmission in Kinmen Island. The possible vector tick responsible for the transmission of *Babesia* infection need to be further identified.

1. Introduction

Babesiosis is a tick-transmitted protozoal infection caused by an intraerythrocytic malaria-like organism that infects a wide variety of wild and domestic animals (Gray et al., 2010). Human infection with *Babesia* parasite is usually acquired via the bite of an infective *Ixodes* tick (Spielman et al., 1985; Hunfeld et al., 2008) or accidentally acquired via blood transfusion (Asad et al., 2009; Alhumaidan et al., 2013). Rarely, vertical transmission is described (Aderinboye and Syed, 2010; Joseph et al., 2012). Since the first case of human babesiosis documented in Yugoslavia (Skrabalo and Deanovic, 1957), numerous cases of human babesial infections have increasingly been reported in Europe and the United States (Zintl et al., 2003; Gray, 2006; Krause et al., 2008; Vannier and Krause, 2012). Reported cases of human babesiosis are rare in eastern Asia, and have recently been reported in Taiwan (Shih et al., 1997), Japan (Saito-Ito et al., 2000), Korea (Kim et al., 2007) and China (Qi et al., 2011; Jiang et al., 2015). The European cases have been attributed to infection with *Babesia* of bovine origin (Zintl et al., 2003). However, *Babesia microti*, a species of rodent origin, has been recognized as the major etiological agent for human babesiosis in the United States (Gray et al., 2010) and East Asia (Shih et al., 1997; Wei et al., 2001; Zhou et al., 2014).

The efficiency of an animal host to serve as reservoir host for a specific pathogen depends mainly on its susceptibility to infect and perpetuate which pathogen. In enzootic regions, small rodents serve as a competent host for transmission and perpetuation of *Babesia* parasites through a natural cycle between rodent hosts and vector ticks. Indeed, rodent-borne *B. microti* was perpetuated in a cycle involving white-footed mouse (*Peromyscus leucopus*) and deer tick (*I. dammini*) in the northeastern and northcentral regions of the United States (Hunfeld et al., 2008). In addition, *B. microti* has long been known to parasitize rodents and ticks in Europe (Hussein, 1980; Alekseev et al., 2003). Although rodent species of

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Rattus coxinga in Taiwan (Shih et al., 1997) and *Apodemus speciosus* in Japan (Wei et al., 2001) have been presumed as the possible reservoir hosts for *B. microti*-like organisms, the reservoir competence for the transmission and perpetuation of *Babesia* parasites in these countries needs to be further identified.

It is assumed that zoonotic agents for babesiosis and Lyme borreliosis share a similar life cycle that depends on rodents and *Ixodes* ticks. Previous evidences reported from the United States, Europe, and Asia had shown that simultaneous infection of *B. microti* and Lyme disease spirochetes (*Borrelia burgdorferi*) could occur in human, rodent hosts, and vector ticks (Krause et al., 2002; Swanson et al., 2006; Zhao et al., 2013). In our previous studies, high prevalence of *Borrelia* spirochetes was detected in rodent host (*R. losea*) and vector tick (*I. granulatus*) collected from Kinmen Island of Taiwan (Shih and Chao, 1998; Chao et al., 2012). It may be that the rodent host of *R. losea* serves as the same reservoir host for the *Babesia* parasites on Kinmen Island. Thus, the objectives of the present study intend to determine the prevalence of *Babesia* infection in rodent hosts by parallel microscopy and PCR assays, and to clarify the genetic identity of *Babesia* parasites by analyzing phylogenetic relationships with other *Babesia* and protozoa species that have been documented in GenBank.

2. Materials and methods

2.1. Epizootiologic survey

Small rodents were trapped and captured at various field sites of four townships on Kinmen Island (Fig. 1). After appropriate anesthetization, whole-blood specimens were obtained by cardiac puncture from 283 rodents. These rodents include 280 brown country rats (*Rattus losea*, Swinhoe), 1 brown rat (*R. norvegicus*, Erxleben) and 2 house shrews (*Suncus murinus*, Linnaeus). The species of trapped rodents was identified to the species level according to the pictorial keys described in "The mammals of Taiwan" (Yu and Lee, 1990).

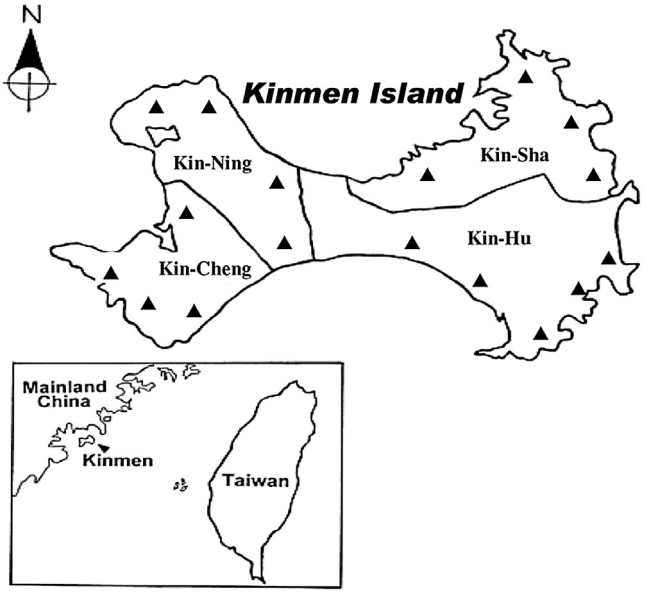

Fig. 1. Map of Kinmen Island and Taiwan, showing the geographic location of Kinmen Island and the collection site (▲) for rodents from four townships on Kinmen Island.

2.2. Diagnosis of Babesia infection by microscopy

Blood specimens collected from each rodent were placed into EDTA-coated blood collection tube (Vacutainer 6457, Becton Dickinson, Taipei, Taiwan) and thin blood smears were made immediately from each rodent's blood. After fixation by methanol, Giemsa-stained thin smears were examined (at least 50 random fields per smear) for the evidence of babesial infection by oil immersion microscopy (Model BX60, Olympus Optical Ltd., Tokyo, Japan). The remaining blood specimens were preserved at deep freezer for further analysis.

2.3. DNA extraction from blood specimens

Total genomic DNA used in this study was extracted from 1 ml blood of each rodent. Briefly, blood samples containing plasma and leukocytes were resuspended with sterile distilled water, the mixed solution was then pelleted by centrifugation (16000 × g for 10 min at 4 °C) (Kawasaki, 1990), and the resulting pellet in a microcentrifuge tube was filled with 200-μl lysing buffer solution supplied in the DNeasy Blood & Tissue Kit (catalogue no. 69506, Qiagen, Hilden, Germany) and then homogenized with an ultrasonic homogenizer (Microson, NY, USA). The homogenate was further processed using a DNeasy Blood & Tissue Kit, as per manufacturer's instructions. After filtration, the eluate (approximately 200-μl) was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (GeneQuant II, Pharmacia Biotech, Uppsala, Sweden).

2.4. Detection of Babesia infection by polymerase chain reaction (PCR)

DNA samples extracted from the blood specimens were used as a template for PCR amplification. For screening of *Babesia* infection, PCR using a piroplasm-specific primer set of Piro-A (5′-AATACCCAATCCTGACACAGGG-3′) and Piro-B (5′-TTAAATACGAATGCCCCCAAC-3′) was performed to amplify the specific fragment encoding the nuclear small subunit ribosomal RNA (18S rRNA) of piroplasms, as described previously (Armstrong et al., 1998). Further identification for *B. microti* infection by PCR using a species-specific primer set of Bab 1 (5′-CTTAGTATAAGCTTTTATACAGC-3′) and Bab 4 (5′-ATAGGTCAGAAACTTGAATGATACA-3′) was also performed for only PCR-positive (using Piro A/B) samples and expected to yield approximately a 240 bp fragment of *B. microti* DNA, as described previously (Persing et al., 1992). All PCR reagents and Taq polymerase were obtained and used as recommended by the supplier (Takara Shuzo Co., Ltd., Japan). Briefly, a total of 0.2-μM (≒10 pmol) of the appropriate primer set and adequate amounts (3–5 μl) of template DNA were used in each 50-μl reaction mixture. The PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700, Applied Bioosystems, Taipei, Taiwan), and the primary amplification (Piro A/B) included 2 min denaturation at 96 °C followed by 35 cycles of the following conditions: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. For species-specific amplification (Bab 1/4), 35 cycles of the same conditions except the annealing at 55 °C for 1 min. Thereafter, amplified DNA products were electrophoresed on 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualized under ultraviolet (UV) light after staining with ethidium bromide. A DNA ladder (1-kb plus, catalogue no. 10787-018, Invitrogen, Taipei, Taiwan) was used as the standard marker for comparison. A negative control of distilled water was included in parallel with each amplification.