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Dynamics, co-infections and characteristics of zoonotic tick-borne pathogens in Hokkaido small mammals, Japan

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ABSTRACT

Many of the emerging infectious diseases originate in wildlife and many of them are caused by vector-borne pathogens. In Japan, zoonotic tick-borne pathogens (TBPs) are frequently detected in both ticks and wildlife. Here, we studied the infection rates of potentially zoonotic species, including *Anaplasma*, *Ehrlichia*, Neoehrlichia and *Babesia* spp., in Hokkaido's most abundant small mammals as they relate to variable extrinsic factors that might affect the infection rates of these pathogens. A total of 412 small mammals including 64 *Apodemus argenteus*, 219 *Apodemus speciosus*, 78 *Myodes rufocanus*, 41 *Myodes rutilus*, 6 *Myodes rex* and 4 *Sorex unguiculatus* were collected from Furano and Shari sites in Hokkaido, Japan, in 2010 and 2011 and were examined by multiplex PCR for TBPs. A reverse line blot hybridization (RLB) was then developed for the specific detection of 13 potentially zoonotic TBPs. A total of 4 TBPs were detected: *Anaplasma* sp. AP-sd, *Ehrlichia muris*, *Candidatus* Neoehrlichia mikurensis and *Babesia microti*. The infection rates were 4.4% (18/412), 1.2% (5/412), 13.1% (54/412) and 17.2% (71/412), respectively. The infection rates of each of the detected TBPs were significantly correlated with host small mammal species. A total of 22 (two triple and 20 double) co-infection cases were detected (5.3%). The most frequent co-infection cases occurred between *Candidatus* N. mikurensis and *B. microti* 68.2% (15/22). Further studies are required to examine human exposure to these zoonotic TBPs in Hokkaido.

1. Introduction

Tick-borne pathogens are maintained in lifecycles that include ticks and animals. Occasionally, these pathogens can be transmitted to humans (de la Fuente et al., 2008), which are generally dead-end hosts. Many TBPs are known to be zoonotic and have been detected in humans. For example, human granulocytic anaplasmosis, human monocytic ehrlichiosis and human babesiosis are caused by the zoonotic tick-borne pathogens *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis* and *Babesia microti*, respectively. In Japan, fever of unknown cause and rickettsiosis-like symptoms have been reported in humans (Gaowa et al., 2013, 2014; Tabara et al., 2007). Unfortunately, the causative agents of these symptoms are rarely determined. Furthermore, several TBPs including members of *Anaplasma*, *Ehrlichia* and *Babesia* species have been detected in Japan (Kawahara et al., 1999, 2004, 1993; Zamoto-Niikura et al., 2012); however, the complex ecologies of many of these TBPs are still not fully understood.

Despite considerable research on tick-borne diseases, there is relatively little information about the ecologies of these pathogens in Japan. The scarcity of information may be in part due to the lack of molecular techniques with high specificity and throughput to detect these pathogens. The use of molecular assays in the identification of these pathogens showed higher specificity and sensitivity than microscopical or serological examinations (Criado-Fornelio,

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2007). Reverse line blot (RLB) is a practical epidemiological tool that can identify those pathogens (Oura et al., 2004) through examination of a large number of samples for many parasitic microbes simultaneously (Kong and Gilbert, 2006). The co-existence of these pathogens in Hokkaido ecosystem could be driven by many variables such as age, gender, season and reservoir host preferences. The objective of this study was to assess infection rates and host affinity, as well as molecular characterization, of TBPs of public health concern among small mammal populations in Hokkaido. We also evaluated involvement of variable extrinsic factors that might affect the infection rates of these pathogens.

2. Material and methods

2.1. Small mammal blood samples and DNA extraction

A total number of 200 Sherman traps baited with oats were placed at 5 m intervals in two grids of 10 × 10. The traps were checked every 24 h during the 3rd to 5th day of each month from May through September of 2010 and during the same period in 2011. Blood samples were collected from 412 trapped small mammals including 219 large Japanese field mice (*Apodemus speciosus*), 64 small Japanese field mice (*Apodemus argenteus*), 78 grey red-backed voles (*Myodes rufocanus*), 41 northern red-backed voles (*Myodes rutilus*), 6 Hokkaido red-backed voles (*Myodes rex*) and 4 long-clawed shrews (*Sorex unguiculatus*) from Furano and Shari sites in Hokkaido, Japan.

Species identification was performed as previously described (Abe et al., 1994; Ohdachi et al., 2009). These small mammals were classified by age into adult and sub-adult groups as previously described (Taylor et al., 2013). Sampling methods were approved by the Animal Care and Use Committee of Hokkaido University (Approval No. JU1105). DNA was extracted from 1 ml of each blood sample using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The obtained DNA samples were kept at −20 °C until analysis.

2.2. Multiplex PCR and reverse line blot (RLB) hybridization

Blood DNA samples from a total of 412 small mammals were examined by multiplex PCR and RLB. The primers Ehr-F (5′-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3′) (Schouls et al., 1999), Ehr-R (5′-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3′) (Bekker et al., 2002), RLB-F2 (5′-GAC ACAGGG AGG TAG TGA CAA G-3′) and RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) (Gubbels et al., 1999; Matjila et al., 2004) were used to amplify a fragment of 460–520 bp from the 16S rRNA gene of *Anaplasma* and *Ehrlichia* and 460–540 bp from the 18S rRNA gene of *Babesia* and *Theileria* species. Multiplex PCR was performed using the PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and QIAGEN Multiplex PCR kits (Qiagen, Hilden, Germany). PCR reactions consisted of 25 μl of 2x QIAGEN Multiplex PCR Master Mix, 1.25 μl of each primer (10 pmol), 2 μl DNA and 18 μl RNase-free water. DNAs from cultured *A. phagocytophilum* (Ameland Netherland strain), *Ehrlichia chaffeensis* and *E. ruminantium* (Welgevonden strain) were used as positive controls and double-distilled water was used as a negative control. The amplification conditions included an initial activation step of 15 min at 94 °C followed by a touch down step of 12 cycles of 30 s at 94 °C, 90 s at 69 °C and 90 s at 72 °C with the annealing temperature decreasing every cycle by 2 °C. This was followed by 40 cycles of 30 s at 94 °C, 90 s at 57 °C and 90 s at 72 °C and a final extension step of 10 min at 72 °C. PCR products were examined by electrophoresis through 1% agarose gel stained with ethidium bromide and visualized by an UV illuminator.

RLB was performed on the PCR products as previously described (Kong and Gilbert, 2006) with modifications. Briefly, a 15 × 15 cm Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated by 20 ml of 16% 1-ethy-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC, Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Subsequently, the membrane was washed gently with milliQ water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, MA, USA). Oligonucleotide probes with C6 amino linker (Table 1) were obtained from Sigma Aldrich Co., LLC, Japan. Five microliters of each 100 pmol/μl probes were diluted in 0.5 M $NaHCO_3$ to a final volume of 170 μl. The slots of the miniblotter were filled with 150 μl of each diluted oligonucleotide and the membrane was incubated at room temperature for 5 min. The membrane was inactivated in 250 ml of 0.1 M NaOH with gentle shaking for 8 min at room temperature, then washed with prewarmed 250 ml of 2 × SSPE/0.1% SDS for 5 min at 60 °C. Then the membrane was directly used or it was stored in a sealed plastic bag with 15 ml of 20 mM EDTA, pH 8.0.

A volume of 10 μl of each PCR product was diluted in 2 × SSPE/0.1% SDS to a final volume of 170 μl, heat-denatured in boiling water for 10 min and immediately cooled on ice. The PCR products were introduced into the miniblotter, which contained the prepared membrane, and hybridized at 60 °C for 1 h. The membrane was washed twice in 250 ml of 2 × SSPE/0.5% SDS for 10 min at 60 °C and then incubated with diluted peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Walyham, MA, USA) for 45 min at 42 °C. The membrane was washed twice with 2 × SSPE/0.5% SDS for 10 min at 42 °C. Finally, the membrane was incubated with 15 ml ImmobilonTM Western Chemiluminescent HRP Substrate (Milipore, Japan) for 5 min at room temperature, and the result was detected by a chemiluminescent imager (Billerica, MA, USA). To strip hybridized PCR products from the membrane for reuse, it was washed twice in prewarmed 1% SDS at 90 °C for 30 min and washed with 250 ml of 20 mM EDTA. The membrane was stored in a plastic bag with 15 ml of 20 mM EDTA at 4 °C until reuse.

2.3. Cloning and sequencing of 16S rRNA and 18S rRNA genes

The result of the RLB was confirmed through cloning of randomly selected 6 and 12 positive PCR products for *Anaplasma*/*Ehrlichia* and *Babesia* spp., respectively. We performed PCR using the KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Osaka, Japan). The same primers as mentioned above were used to amplify a segment of *Anaplasma*/*Ehrlichia* spp. 16S rRNA and *Babesia* spp. 18S rRNA. Each PCR reaction consisted of 2.5 μl of 10× KOD-Plus-Neo buffer, 2.5 μl of dNTPs (2 mM), 1.5 μl of 25 mM $MgSO_4$, 0.75 μl of each primer (10 pmol), 1 μl of DNA (100 ng/μl) and 0.5 μl (1 U/μl) of KOD-Plus-Neo DNA polymerase. The reaction conditions were 94 °C for 2 min and 40 cycles of 98 °C for 10 s, 57 °C for 30 s and 68 °C for 30 s, followed by a final extension at 68 °C for 7 min.

Nine microliters of each PCR product were incubated for 10 min at 60 °C with 1 μl of 10 × A-attachment mix (Toyobo Co., Ltd.). The A- tailed PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) as described in the manufacturer's quick protocol manual. Recombinant plasmids were transformed into competent *Escherichia coli* DH5α cells (Nippon Gene, Tokyo, Japan). Five colonies per sample were selected and screened by PCR for recombinants. Five microliters of each PCR product were purified by incubation with 4 μl Exosap mix (USB Co., Cleveland, OH, USA) for 30 min at 37 °C followed by 15 min at 80 °C. The purified PCR products were subjected to sequencing reactions using the Big- Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and cleaned by Agencourt CleanSEQ (Agencourt Bioscience Co., Beckman Coulter, Beverly, MA, USA) according to the manufacturer's instructions. The cleaned products