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Original Article

Serological and molecular prevalence of equine piroplasmosis in Western Java, Indonesia

Arifin Budiman Nugraha[a,b], Umi Cahyaningsih[b], Amrozi Amrozi[b], Yusuf Ridwan[b], Srihadi Agungpriyono[b], Dharmawaty Muhammad Taher[c], Azirwan Guswanto[a,d], Sambuu Gantuya[a], Dickson Stuart Tayebwa[a], Bumduuren Tuvshintulga[a], Thillaiampalam Sivakumar[a], Naoaki Yokoyama[a], Ikuo Igarashi[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[b] Faculty of Veterinary Medicine, Bogor Agricultural University, Jl. Agatis Kampus IPB Dramaga Bogor, 16680 Indonesia
[c] Faculty of Teacher Training and Education Program of Biology Education, Khairun University, Jl. Bandara Babullah, P.O. Box 53, Ternate 97728, North Maluku, Indonesia
[d] Balai Veteriner Subang (DIC Subang), Jl. Terusan Garuda 33/11 Blok Werasari Dangdeur, Subang 41212, Indonesia

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ABSTRACT

Equine piroplasmosis is an economically significant disease caused by *Theileria equi* and *Babesia caballi*, which are tick-borne hemoprotozoan parasites. Infections with these parasite species had never been reported in horses in Indonesia. The aim of the present study was to investigate the prevalence of *T. equi* and *B. caballi* in horses reared in parts of Western Java, Indonesia. Blood samples were collected randomly from 235 horses in four different districts (Bandung, Depok, Tangerang, and Bogor) in Western Java, Indonesia. Thin blood smears prepared from the sampled animals were stained by Giemsa and observed under a light microscope. Serum samples prepared from blood were screened by enzyme-linked immunosorbent assays (ELISAs) based on recombinant forms of EMA-2 and BC48 antigens to determine the seroprevalence of *T. equi* and *B. caballi*, respectively. DNA samples extracted from the same blood samples were screened by *EMA-2* and *BC48* gene-based nested polymerase chain reaction (nPCR) assays for *T. equi* and *B. caballi* infections, respectively. Of 235 surveyed animals, five (2.1%) and 15 (6.4%) were seropositive for *T. equi* and *B. caballi*, respectively, whereas one and four horses were nPCR-positive for *T. equi* and *B. caballi*, respectively. All of the surveyed animals were negative for *T. equi* and *B. caballi* by microscopy. The *T. equi EMA-2* and *B. caballi BC48* gene fragments amplified by the nPCR assays were cloned, sequenced, and subjected to bioinformatic and phylogenetic analyses. The *T. equi EMA-2* gene sequence from an Indonesian horse was identical to sequences from Florida and Washington strains and clustered together with these sequences in phylogeny. On the other hand, four Indonesian *BC48* gene sequences shared 99.8–100% identity scores. This present study is the first to report *T. equi* and *B. caballi* in horses in Indonesia. Our findings highlight the need for monitoring horses in Indonesia for clinical piroplasmosis caused by *T. equi* and *B. caballi*.

1. Introduction

Equine piroplasmosis is an economically significant infectious disease in horses. The disease has been reported worldwide except for a few countries, including Japan, the United States, and Australia (OIE, 2014). Equine piroplasmosis is a major obstacle to the international movement of horses for trade and sport. Therefore, controlling this disease is a priority in the equine industry. The causative agents of equine piroplasmosis are *Theileria equi* and *Babesia caballi*, which are intraerythocytic hemoprotozoan parasites. Both *T. equi* and *B. caballi* are transmitted by ixodid ticks, mainly those belonging to the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus*, which are distributed worldwide (APHIS, 2018). Once infected, horses remain carriers of *T. equi* and *B. caballi*, which can be transmitted via ticks to other susceptible host animals. Horses infected with *T. equi* remain seropositive for their entire lives, while those infected with *B. caballi* remain

* Corresponding author at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13 Inada-cho, Obihiro 080-8555, Japan.
E-mail address: igarcpmi@obihiro.ac.jp (I. Igarashi).

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seropositive for up to four years (Zweygarth et al., 1996). Equine piroplasmosis caused by *B. caballi* and *T. equi* is characterized by fever, anemia, icterus, and hemoglobinuria (Zobba et al., 2008). Deaths are common if animals are not treated with the proper antiprotozoan agents in the early stage of the clinical disease. The clinical signs associated with *B. caballi* are usually milder than those induced by *T. equi* (de Waal, 1992).

The microscopic examination of a Giemsa-stained thin blood smear is a widely used diagnostic method for detecting *B. caballi* and *T. equi*. However, the use of this technique is limited by its law of specificity and sensitivity (Böse et al., 1995). Therefore, PCR assays with high sensitivity and specificity are preferred over microscopy to detect these parasite species in epidemiological surveys, which often target carrier animals (Alhassan et al., 2005; Nicolaiewsky et al., 2001). As PCR assays detect only active infections, serological diagnostic tools, such as the enzyme-linked immunosorbent assay (ELISA) (Munkhjargal et al., 2013; Xuan et al., 2001) and the immunofluorescence antibody test (IFAT), are widely used to detect parasite-specific antibodies and, thereby, estimate seroprevalence rates, which indicate the risk of exposure to *B. caballi* and *T. equi*. Additionally, previous studies demonstrated that more informative data can be generated if a combination of molecular and serological diagnostic methods is used than if single techniques are employed (Baptista et al., 2013; Mahmoud et al., 2016; OIE, 2014; Rosales et al., 2013; Seo et al., 2011). Indonesia is a Southeast Asian country with a horse population of approximately 430,000. Various infectious diseases prevalent among horses, such as surra (Payne et al., 1991) and equine distemper, or strangles (Hidayat and Alhadi, 2012), represent significant threats to the economic success of the equine industry in Indonesia. However, *B. caballi* and *T. equi* infections have never been investigated in horses in that country. The aim of the present study was to investigate the prevalence of *B. caballi* and *T. equi* in horses in Western Java, Indonesia, using serological and molecular diagnostic tools.

2. Materials and methods

2.1. Sample collection

This study was carried out in four regions of Western Java, Indonesia: Bandung, Depok, Tangerang, and Bogor (Fig. 1). Blood samples were collected randomly from 235 horses. All samples were from apparently healthy horses. The horses investigated in the present study were thoroughbreds, warmbloods, or crossbreeds. From each animal, a blood sample of approximately 3 ml was collected into a vacutainer tube with or without Ethylenediaminetetraacetic acid (EDTA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In addition, thin blood smears prepared from the sampled animals were stained with Giemsa and observed under a light microscope. Serum samples were prepared from blood samples collected in vacutainer tubes without EDTA. DNA samples were extracted from 200 µl of whole blood collected in EDTA-coated tubes using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.

2.2. Enzyme-linked immunosorbent assays

ELISAs for detecting *B. caballi* and *T. equi* antibodies in equine serum samples from Indonesia were conducted using merozoite antigen 2 (EMA-2) and a 48 kDa merozoite rhoptry protein (BC48), respectively. The recombinant EMA-2 and BC48 were expressed and purified as described previously (Huang et al., 2003; Ikadai et al., 1999). ELISA was performed using these recombinant antigens as described by Xuan et al. (2001). Briefly, ELISA plates were coated with 100 µl of EMA-2 or BC48 antigens at a concentration of 2 µg/ml in a carbonate-bicarbonate buffer (50 mM, pH 9.6) and stored at 4 °C overnight. The plates were washed once with 0.05% Tween 20-PBS (PBS-T) and then 100 µl of blocking solution (PBS supplemented with 3% skim milk). After washing once with PBS-T, 100 µl of each serum sample diluted to 1:100 with blocking solution was added and incubated for 1 h at 37 °C. The plates were washed six times with PBS-T, and then 100 µl of the Anti-Horse IgG (whole molecule) peroxidase conjugated (Sigma-Aldrich, St. Louis, MO, USA) as a secondary antibody was diluted to 1:5000 with blocking solution and incubated for 1 h at 37 °C. After washing six times, 100 µl of a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg/ml of 2,2′-azide-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich), and 0.03% of 30% H_2O_2] was added to each well and incubated for 30 min at room temperature (RT). The optical density (OD) was measured at a wavelength of 415 nm. Positive and negative controls were previously prepared from horses experimentally infected with *T. equi* or *B. caballi*. Ten negative controls used in this study were from uninfected horses bred in Japan and confirmed to be negative for *T. equi* and *B. caballi* by ELISA and IFAT. The cutoff value was set as the sum of the mean OD value of the 10 negative sera and the threefold standard deviation. A serum sample was considered positive if the OD value was greater than the cutoff.

2.3. PCR assays

Nested PCR (nPCR) assays targeting *EMA-2* and *BC48* genes were employed to detect *T. equi* (*EMA-2* nPCR) and *B. caballi* (*BC48* nPCR), respectively. The primers for the *EMA-2* nPCR assay were designed in the present study, while previously described primer sets were used to conduct the *BC48* nPCR (Table 1, Battsetseg et al., 2001). Briefly, 20 µl of PCR reaction mixture containing 2 µl of the DNA template, 4 µl of 5 × SuperFi Buffer (*Invitrogen*, Carlsbad, CA, *USA*), 2 µl of GeneAmp® dNTP Mix (Applied Biosystems, Streetsville, Ontario Canada), 0.2 µl of 0.02 U/µL Platinum SuperFi Polymerase (Invitrogen), 1 µl of 10 µM outer forward and reverse primers (Table 1), and 9.8 µl of distilled water was prepared. After an initial enzyme activation step at 98 °C for 30 s, the reaction mixture was subjected to 40 cycles, each of which included a denaturation step at 98 °C for 10 s, an annealing step at 62.8 °C (*T. equi*) or 72 °C (*B. caballi*) for 10 s, and an extension step at 72 °C for 30 s. After a final extension at 72 °C for 10 min, 1 µl of the first PCR product was transferred to a new PCR tube containing a reaction mixture similar to that of the first PCR except for the primers, which were replaced with inner forward and reverse primers (Table 1). The cycling condition for the second round of PCR was similar to that for the first round, except that the annealing temperature was set at 72 °C (*T. equi*) or 70.3 °C (*B. caballi*). The PCR products were then subjected to agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Bands detected at approximately 221 bp and 454 bp were considered positive for *T. equi* and *B. caballi*, respectively.

2.4. DNA cloning and sequencing

All of the PCR amplicons from the *EMA-2* and *BC48* nPCR assays were subjected to cloning and sequencing. Briefly, after gel electrophoresis, PCR amplicons were purified using NucleoSpin® Gel and a PCR Clean-up Kit (MACHEREY-NAGEL, Duren, Germany), ligated to a pCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), transformed into TOP 10 *E. coli* cells (Invitrogen), and plated onto Luria-Bertani (LB) agar plates (Invitrogen). Three clones were selected for each PCR amplicon and cultured in LB broth. The plasmids were extracted using NucleoSpin® Plasmid QuickPure (MACHEREY-NAGEL, Duren, Germany) and subjected to sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Sequence and phylogenetic analyses

The *EMA-2* and *BC48* sequences were initially analyzed using the basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast.cgi). The EMBOSS needle program (http://emboss.