Veterinary Parasitology 212 (2015) 161–167

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon Island, the Philippines

Nyamsuren Ochirkhuu[a,b], Satoru Konnai[a], Claro N. Mingala[c], Tomohiro Okagawa[a], Marvin Villanueva[c], Flor Marie Immanuelle R. Pilapil[d], Shiro Murata[a], Kazuhiko Ohashi[a,*]

[a] Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku-Kita 18 Nishi 9 chome, Sapporo 060-0818, Japan
[b] School of Veterinary Medicine, Mongolian University of Life Science, Khan-Uul district, Zaisan 17042, Ulaanbaatar, Mongolia
[c] Philippine Carabao Center National Headquarters and Genepool, Science City of Munoz, 3120 Nueva Ecija, Philippines
[d] Hacienda Macalauan Brgy. Mabacan, Calauan 4012, Laguna Philippines

ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form 19 May 2015
Accepted 22 May 2015

Keywords:
Amarginale
B. bovis
B. bigemina
Theileria
T. evansi
Philippines

ABSTRACT

In the Philippines, vector-borne disease is one of the important problems in the livestock industry. To elucidate the epidemiology of vector-borne diseases in cattle on Luzon Island, the Philippines, the prevalence of five protozoan agents was assessed by polymerase chain reaction. Out of the 339 samples, 324 (95.5%), 154 (45.4%), 209 (61.6%), 140 (41.3%), and 2 (0.6%) were positive for *Anaplasma marginale*, *Babesia bigemina*, *Babesia bovis*, *Theileria* spp., and *Trypanosoma evansi* infections, respectively. Mixed infections were detected in 290 (85.5%) samples, of which 115 (33.9%) had two pathogens, 144 (42.5%) had three pathogens, and 31 (9.1%) had four kinds of pathogens. *16S rRNA* gene was 100% identical in *A. marginale* compared with the same lineage across the world. *B. bovis RAP-1* and *B. bigemina AMA-1* genes were identical with 92.27%–100% and 97.07%–100% sequences, respectively, in the database (Asian isolates). *MPSP* genes of *Theileria* spp. were 83.51%–100% identical with the one another. Phylogenetic analysis showed that they belong to the groups of *T. sergenti* and *T. buffeli*. Positive rates of the tick-borne pathogens were extremely high in this area. These findings provide vital information that can be used for the planning and execution of effective control measures for vector-borne diseases in the Philippine cattle industry.

1. Introduction

Vector-borne diseases that include anaplasmosis, babesiosis, theileriosis, and trypanosomiasis often cause significant economic loss in cattle production, particularly in the tropical and subtropical regions of the world. Furthermore, these infections can affect the international trade of cattle. In the Philippines, vector-borne disease is one of the important problems in the cattle industry.

Anaplasma marginale is a rickettsial gram-negative, intraerythrocyte pathogen, and quite host-specific infecting only ruminants and primarily causing disease in cattle (Kocan et al., 2010). *A. marginale* can be mechanically and congenitally transmitted by biting flies and most tick species. The disease is characterized by fever and general depression followed by weight loss and progressive anemia and icterus (Minjauw and McLeod, 2003). Both cattle and male ticks develop persistent *A. marginale* infections, and thus serve as reservoirs of the infection for mechanical and biological transmission of the pathogen. Bovine anaplasmosis is widely distributed worldwide, particularly in the United States, Europe, Latin America, Africa, and Asia including the Philippines (Mingala et al., 2009; Kocan et al., 2010).

Babesia bovis and *B. bigemina* are tick-borne intraerythrocyte protozoan parasites and cause a great economic impact on the cattle industry. The main vectors of these pathogens are ticks from the genus *Boophilus* and *Rhipicephalus* (Bock et al., 2004). Cattle with babesiosis usually show severe clinical signs that include continuous fever, high parasitemia, anemia, icterus, and often hemoglobinuria. Without treatment, the mortality rates are very high (30% for *B. bigemina*, 70–80% for *B. bovis*) (Minjauw and McLeod, 2003).

Theileriosis is also a tick-borne, intraerythrocyte protozoan disease of domestic and wild animals. There are five species of *Theileria*: *T. parva*, *T. annulata*, *T. taurotragi*, *T. velifera*, and the group of *T. Sergenti*, *T. Buffeli*, and *T. Orientalis.* All these species have been found in cattle (Parthiban et al., 2010). The disease is character-

* Corresponding author. Fax: +81 11 706 5217.

http://dx.doi.org/10.1016/j.vetpar.2015.05.019
0304-4017/

Table 1
Primer sequences in this study.

Pathogen	Target gene	Oligonucleotide sequence (5'–3')	Reference
A. marginale	*16S rRNA*	1st Fw :AGAGTTTGATCCTGGCTCAG Rev:ACGGCTACCTTGTTACGACTT 2nd Fw:TACGCAGCTTGCTGCGTGTATG Rev:GCCCTTCTGTTAAGAAGGATCTAG	Parola et al. (2000); Adrian et al. (2012)
B. bovis	*RAP-1*	1st Fw:CACGAGCAAGGAACTACCGATGTTGA Rev:CCAAGGACCTTCAACGTACGAGGTCA	Figueroa et al. (1993) Figueroa et al. (1993)
B. bigemina	*AMA-1*	Fw: TACTGTGACGAGGACGGATC Rev:CCTCAAAAGCAGATTCGAGT	Sivakumar et al. (2012)
Theileria spp.	*MPSP*	Fw:CACGCTATGTTGTCCAAGAG Rev:CCTCAAAAGCAGATTCGAGT	Kakuda et al. (1998)
T. evansi	*RoTat 1.2*	Fw:GCGGGGTGTTTAAAGCAATA Rev:ATTAGTGCTGCGTGTGTTCG	Claes et al. (2004)

ized by fever, lymphadenopathy, leukopenia, anorexia, depression, anemia, jaundice, weight loss, and sometimes death. Among these species, the group of *T. Sergenti*, *T. Buffeli*, and *T. orientalis* is transmitted by ticks from the genera *Amblyomma*, *Rhipicephalus*, and *Haemaphysalis*, which are mainly found in Asia and Australia, and are usually benign but can cause heavy losses in the cattle industry (Sugimoto and Fujisaki, 2002; Minjauw and McLeod, 2003).

Trypanosoma evansi is a causative agent of surra in domestic animals and is mechanically transmitted by biting flies (Lun and Desser, 1996; Reid, 2002; Sumba et al., 1998; Otte and Abuabara, 1991). Surra is a serious problem in the livestock industry in the Philippines (Reid, 2002).

The Philippines is an agricultural country located in Southeast Asia, and several vector-borne diseases have been reported in the livestock populations. In previous surveys conducted in the Philippines, the prevalence of *B. bigemina, A. marginale*, and *T. evansi* in the water buffalo were 10.3%, 4.4%, and 2.9%, respectively (Konnai et al., 2008; Mingala et al., 2009). More recently, surveys for tick-borne pathogens have been conducted in Cebu Island (Ybañez et al., 2013a,b). However, the prevalence of these diseases and the genetic diversity of these pathogens have not been studied yet in Luzon Island, the main and largest island in the Philippines. The aims of this study were to detect various bovine tick-borne pathogens that include *A. marginale*, *B. bigemina*, *B. bovis, Theileria* spp., and *T. evansi* and to understand the genetic diversity of these pathogens.

2. Materials and methods

2.1. Blood sample collection

Three hundred thirty-nine bovine blood samples were collected from two dairy farms on Luzon Island of the Philippines in November and December 2012. Blood (5 ml) was collected from the jugular vein using a BD K3EDTA Vacutainer® tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The blood samples were stored at 4 °C until DNA extraction.

2.2. DNA extraction

Genomic DNA was extracted from the blood samples using a Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Total DNA was diluted with 100 μl conservation buffer and stored at −30 °C until further use.

2.3. Diagnostic polymerase chain reaction assays for vector-borne pathogens detection

The oligonucleotide sequences of polymerase chain reaction (PCR) primers used in the present study are shown in Table 1. *A. marginale* and *B. bovis* were screened using a nested PCR assay based on *16S rRNA* (Ybañez et al., 2012) and *rhoptry-associated protein* (*RAP*)*-1* (Figueroa et al., 1993), respectively. *B. bigemina, Theileria* spp., and *T. evansi* were detected using single-step PCR methods based on *apical membrane antigen* (*AMA*)*-1* (Sivakumar et al., 2012), *major piroplasm surface protein* (*MPSP*) (Kakudai et al., 1998) and *RoTat1.2* (Claes et al., 2004) genes, respectively. All PCR assays were conducted as previously described with slight modifications. Briefly, 1.5 μl (100 ng) of a DNA sample was added to 28.5 μl of reaction mixture that comprised 3 μl of 10 × buffer (Takara Bio Inc., Shiga, Japan), 2.4 μl of dNTPs (Takara Bio Inc.,), 2 μl of 10 p μM of each forward and reverse primers (Hokkaido System Science Co.,Ltd., Sapporo, Japan), 0.1 μl of DNA Taq polymerase (Takara Bio Inc.,), and 21.5 μl of double distilled water. PCR amplifications were performed under the following thermal cycle condition: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at each optimal temperature for 30 s, extension at 72 °C for 1 min, and a final synthesis at 72 °C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The identities of the amplified PCR products (expected size of approximately 875 bp, 356 bp, 211 bp, 852 bp, and 205 bp for *A. marginale*, *B. bovis, B. bigemina, Theileria* spp., and *T. evansi*, respectively) were confirmed by electrophoresis on a 1.5% agarose gel and visualized under UV light.

2.4. DNA cloning and sequencing

After the gel electrophoresis, eight positive samples from each pathogen were subjected to sequencing analysis. PCR products were extracted using FastGene gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The extracted PCR products were ligated into the pGEM-T Easy vector (Promega), and the plasmid was introduced into the *E. coli* strain DH5α (Takara Bio Inc.,) and plated on a Luria–Bertani (LB) agar (Invitrogen, Carlsbad, CA, USA) and cultured in an LB broth (Invitrogen). The plasmid DNAs from the positive clones were extracted from the LB culture using FastGene Plasmid Mini Kit (Nippon Genetics). The sequencing amplifications of the plasmids were performed using the GeneAmp PCR System 9700 (Applied Biosystems). The quality of the plasmids was checked with NanoDrop 8000 analytic equipment (Thermo Scientific, Wilmington, DE, (USA). Finally, the nucleotide sequences of the amplified plasmids were determined using CEQ8000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA).

2.5. Phylogenetic and homology analyses

All of the identified pathogens were initially analyzed using the Bio-Edit program and BLAST application. Phylogenetic trees of the present study were constructed with database sequences in the GenBank. Percent similarities of the amino acid sequences were computed using the MEGA 6 program.