Ticks and Tick-borne Diseases 4 (2013) 235–241

Contents lists available at SciVerse ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Genetic diversity within *Theileria orientalis* parasites detected in Sri Lankan cattle

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ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form
16 November 2012
Accepted 16 November 2012
Available online 18 January 2013

Keywords:
Theileria orientalis
Cattle
Sri Lanka
MPSP gene
Genotypes

ABSTRACT

In the present study, we investigated the genetic diversity of *Theileria orientalis* parasites circulating among Sri Lankan cattle. Nucleotide sequence analysis of the major piroplasm surface protein (MPSP) gene fragments amplified from *T. orientalis*-positive DNA samples (from bovine blood) revealed the presence of 4 parasite genotypes. The genotypes consisted of types 1, 3, 5, and 7. Phylogenetic analysis indicated that the Sri Lankan *MPSP* sequences were closely related to those reported from Vietnam (types 3 and 5), Mongolia (types 1 and 5), Thailand (types 1, 5, and 7), and Japan (type 7). Subsequently, genotype-specific PCR assays determined that the most common genotype was type 7, followed by types 5, 3, and 1. Genotype 7 has been reported to be involved in disease outbreaks in India. Therefore, preventive and control measures are essential to avoid potential economic losses due to *T. orientalis* infection in Sri Lanka. This is the first report that describes the genetic diversity of *T. orientalis* circulating among Sri Lankan cattle.

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Introduction

Hemoprotozoan parasites that infect cattle are of worldwide clinical and economic importance (Uilenberg, 1995; Wagner et al., 2002). Many different species of *Babesia*, *Theileria*, and *Trypanosoma* parasites are known to have a detrimental impact on cattle health (McCosker, 1981). Among such parasites, a group of non-lymphoproliferative *Theileria* parasites, which include *T. orientalis*, *T. sergenti*, and *T. buffeli*, is generally considered to have low pathogenicity in cattle (Minami et al., 1980). Some authors suggested that these 3 parasites are the same, while others have the opinion that they must be considered as different species (Fujisaki et al., 1994; Kakuda et al., 1998; Uilenberg et al., 1985). We use a common name *T. orientalis* to describe this benign *Theileria* group. Although the parasite is considered benign, *T. orientalis* disease outbreaks that have caused substantial economic losses were reported from different regions of the world (Aparna et al., 2011; McFadden et al., 2011). *T. orientalis* parasite populations can be divided into well-defined genotypes, some of which lack antigenic cross reactivity between each other (Onuma et al., 1998; Yokoyama et al., 2012). Hence, susceptible cattle can be repeatedly infected with different *T. orientalis* genotypes.

Initial studies divided *T. orientalis* parasites into 4 types, namely the Ikeda, Chitose, Thai, and Buffeli types (Kakuda et al., 1998; Sarataphan et al., 1999) using limited numbers of major piroplasm surface protein (MPSP) gene sequences. However, later studies that generated more numbers of *MPSP* sequences speculated that genetic differences might exist within the above genotypes. Therefore, Kim et al. (1998) proposed a different approach, by which 6 genotypes designated types 1–6, were identified based on *MPSP* gene sequences. Subsequently, 5 more genotypes, i.e., types 7, 8, N1–N3, have been added to the list of previously described genotypes (Jeong et al., 2010; Khukhuu et al., 2011). The latter classification method has been employed to analyze the *T. orientalis* population structure in different countries (Khukhuu et al., 2011; Yokoyama et al., 2011; Altangerel et al., 2011a,b).

Sri Lanka is an agriculturally rich country, and the livestock industry has been identified as a potentially important sector

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http://dx.doi.org/10.1016/j.ttbdis.2012.11.009

Table 1
Theileria orientalis genotypes detected by analysis of *MPSP* sequences determined from parasite-positive DNA samples.

District	No. of *MPSP* gene sequences	Genotypes			
		1	3	5	7
Nuwara Eliya	36	4	4	21	7
Polonnaruwa	13	0	4	7	2
Ampara	13	1	3	3	6
Jaffna	09	1	1	0	7
Total	71	6	12	31	22

for future development. If dairy cattle milk production can be improved, the large sums of foreign currency that are currently being spent on importing milk products could be diverted to issues of national importance. However, the spread of infectious diseases in Sri Lankan cattle is one of the major obstacles hindering milk production in dairy herds.

Our recent PCR-based study conducted among Sri Lankan cattle populations indicated that *T. orientalis* was the predominant parasite, and over 50% of the animals surveyed were infected with this parasite (Sivakumar et al., 2012). In one of the sampling locations, the prevalence of *T. orientalis* was recorded as 98.8%, indicating that almost all of the cattle populations in that area were infected with this pathogen. Different virulence characteristics have been reported for *T. orientalis* genotypes (Kamau et al., 2011). For instance, a recent study has indicated that more clinical cases of *T. orientalis* were associated with type 2 (Ikeda type) than type 1 (Chitose type) (Eamens et al., 2013). The involvement of type 7 in clinical theileriosis was also previously observed in India (Aparna et al., 2011). Therefore, the detection of *T. orientalis* genotypes is important to determine the clinical significance of this pathogen in Sri Lanka. In addition, better understanding about the genetic diversity in *T. orientalis* parasites is an important prerequisite for devising effective immune control strategies in the future, as the previous studies showed that subunit vaccines based on MPSP could be effectively used for the control of clinical theileriosis (Onuma et al., 1998). In the present study, therefore, we aimed to determine the number of *T. orientalis* genotypes circulating among Sri Lankan cattle. Although the nucleotide sequences of the *MPSP* genes collected from the *T. orientalis*-positive blood DNA samples may provide preliminary information on the genotypes, the actual prevalence of such genotypes cannot be estimated accurately, as the mixed infections with different genotypes are commonly observed. Therefore, parasite genotype-specific PCR assays were developed and employed to determine the prevalence of each genotype detected by DNA sequence analysis.

Materials and methods

DNA samples

In Sri Lanka, 316 blood samples were collected from cattle populations bred in 4 different districts namely the Nuwara Eliya ($n = 83$), Polonnaruwa ($n = 84$), Ampara ($n = 88$), and Jaffna ($n = 61$) in May–June 2011 using EDTA-coated Vacutainer tubes (NIPRO, Osaka, Japan). All the sampled animals were clinically normal and over one year of age. Subsequently, DNA samples were prepared from whole blood as described in our previous report (Sivakumar et al., 2012) using a DNA extraction kit (Qiagen, Hilden, Germany). Among these DNA samples, 169 (Nuwara Eliya $n = 82$, Polonnaruwa $n = 26$, Ampara $n = 38$, and Jaffna $n = 23$) were positive for *T. orientalis* (Sivakumar et al., 2012) and used in the present study.

PCR amplification, cloning, and sequencing

A previously described MPSP-PCR assay was used to amplify a part of the hypervariable region of the *MPSP* gene (776 bp) from randomly selected *T. orientalis*-positive DNA samples obtained from each district (Table 1), using a set of forward (5′-CTTTGCCTAGGATACTTCCT-3′) and reverse (5′-ACGGCAAGTGGTGAGAACT-3′) primers (Ota et al., 2009). After agarose gel electrophoresis, *T. orientalis*-specific PCR products were purified (Alquick Gel Extraction Kit, Qiagen), ligated to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA), transformed into Top 10 *E. coli* competent cells (TOP 10, Invitrogen), and then plated onto Luria Broth (LB) agar plates (Invitrogen) that contained 50 μg/ml X-gal (Wako, Osaka, Japan). After incubation overnight at 37 °C, individual white colonies were picked and cultured in LB broth (Invitrogen). Plasmid DNA was extracted from the bacterial cultures (QIAprep Spin Miniprep kit, Qiagen), and the nucleotide sequences of the inserts were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Construction of phylogenetic trees

The *MPSP* gene sequences generated in the present study ($n = 71$), together with 6 other Sri Lankan sequences that had been determined in our previous investigation (AB690866–AB690871) (Sivakumar et al., 2012), were analyzed using GENETYX 7.0 software (GENETYX, Tokyo, Japan). Selected numbers of Sri Lankan *MPSP* gene sequences ($n = 37$) together with those reported from other countries ($n = 47$) were used to construct a neighbor-joining phylogenetic tree using the MAFFT program (Katoh et al., 2002). Bootstrap values were calculated using the same software.

Genotype-specific PCR assays

The specificities of the previously described (Yokoyama et al., 2011) and newly developed genotype-specific PCR assays were evaluated using DNAs derived from field samples and plasmids containing *MPSP* gene fragments derived from *T. orientalis*-positive Sri Lankan cattle. The primers for the genotype-specific PCR assays developed in the present study were carefully designed using specific and conserved regions of *MPSP* sequences as described previously (Yokoyama et al., 2011). Composition of PCR reaction mixtures and the cycling conditions of all the genotype-specific PCR

Table 2
List of primers used for the detection of types 1, 3, 5, and 7 of *T. orientalis*.

Type	Primer[a]	Primer sequence (5′–3′)	Annealing temperature (°C)	Size of amplicon (bp)	Reference
1	F	TTGCCTAGGATACTTCCTCATCG	64	559	Yokoyama et al. (2011)
	R	TGCGGTGTATTTGGCCTTC			
3	F	CCCTTCAAGGTTAAGAGT	58	287	Present study
	R	ACGGCAAGTGGTGAGAACT			Ota et al. (2009)
5	F	CAGTCAATGCAACAAAACCCGA	56	424	Yokoyama et al. (2011)
	R	CTTTTTAGGATCACCGACATCCAG			
7	F	GGAAAAGAAAGACCTCGATGTG	65	232	Present study

[a] F, forward primer; R, reverse primer.

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