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Research paper

Molecular characterisation of *Theileria orientalis* in imported and native bovines from Pakistan

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ABSTRACT

The epidemiological aspects of *Theileria orientalis* in Pakistan are unknown; therefore, investigations using sensitive and precise molecular techniques are required. This study reports the first molecular characterisation of *T. orientalis* detected from imported (*Bos taurus*) and native cattle (*Bos indicus* × *Bos taurus*) and buffaloes (*Bubalus bubalis*) selected from four districts of Punjab, Pakistan. DNA samples from blood ($n = 246$) were extracted and tested using conventional PCR utilising the major piroplasm surface protein (*MPSP*) gene and multiplexed tandem PCR (MT-PCR). *Theileria orientalis* DNA was detected (15%; 22/147) only in imported cattle by conventional PCR, whereas 24.5% (36/147), 6% (3/50) and 6.1% (3/49) of the imported cattle and native Pakistani cattle and buffaloes, respectively were test-positive for *T. orientalis* using MT-PCR. Using MT-PCR, the prevalence of *T. orientalis* was significantly higher ($P < 0.0001$) in imported cattle compared to that of detected in native Pakistani bovines. The prevalence of *T. orientalis* and DNA copies of *chitose* and *ikeda* were significantly higher ($P < 0.05$) in imported cattle than those detected in native Pakistani bovines. DNA sequencing of amplicons of the conventional PCR revealed the presence of *buffeli*, *chitose* and *ikeda* genotypes of *T. orientalis*. Phylogenetic analysis revealed that the *MPSP* sequences of *buffeli*, *chitose* and *ikeda* from imported cattle were closely related to those sequences reported previously from Australia and other regions. This study provides the first survey of *T. orientalis* infection in imported and native bovines in Pakistan, and highlights the need for future studies to understand the spread of transboundary animal diseases.

1. Introduction

Piroplasmosis is a tick-transmitted disease caused by the two closely related genera, *Theileria* and *Babesia*, and it affects various domestic and wild ruminants, leading to substantial economic losses in tropical and sub-tropical regions (Uilenberg, 1995; Makala et al., 2003). To date, *Theileria parva, T. annulata, T. taurotragi, Theileria* sp. (buffalo), *T. mutans, T. velifera* and *T. orientalis* complex, are known to infect bovines globally (Sivakumar et al., 2014a).

Recently, oriental theileriosis, caused by the two pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis*, has emerged as an economically important tick-borne disease of cattle in Australia and New Zealand (Izzo et al., 2010; Kamau et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014, 2015a- b; Gebrekidan et al., 2015, 2016a). Based on the sequence analysis of the major piroplasm surface protein (*MPSP*) gene, 11 genotypes: *chitose* (type 1), *ikeda* (type 2), *buffeli* (type 3–8) and N (1–3) have been reported (Sivakumar et al., 2014a). The disease is generally characterised by pyrexia, haemolytic anaemia, productivity losses, abortions and/or mortality in dairy and beef cattle (Izzo et al., 2010; Islam et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014).

In Pakistan, tropical theileriosis caused by *T. annulata* is one of the most important tick-borne diseases of bovines and to date, there has been no report of *T. orientalis* in bovines from Pakistan (Jabbar et al., 2015). However, *T. orientalis* genotypes have been reported in buffaloes and cattle from neighbouring countries, including India (Aparna et al., 2011), Iran (Ghaemi et al., 2012) and Sri Lanka (Sivakumar et al., 2013) as well as South-east Asian countries (Sivakumar et al., 2014a).

Owing to an increased demand for milk and milk products for a rapidly growing population in Pakistan, the governmental and private dairy sectors, imported high-milk producing breeds of cattle (Holstein and Friesian) from Australia, a country known to be endemic for oriental theileriosis (Izzo et al., 2010; Islam et al., 2011; Perera et al., 2013; Gebrekidan et al., 2015). However, the occurrence of *T. orientalis* in native cattle and buffaloes and the potential role of the imported cattle as a source of infection are not studied. Therefore, the present study was designed to detect, differentiate and to quantitate the *T. orientalis*

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genotypes *chitose* and *ikeda* in imported and native bovines in Pakistan using conventional and multiplexed tandem PCR.

2. Materials and methods

2.1. Study location and animal characteristics

Blood samples were collected from either imported Holstein and Friesians cattle (*Bos Taurus*) or native Pakistani cattle (*Bos taurus* × *Bos indicus*) ($n = 197$) and buffaloes (*Bubalus bubalis*) ($n = 49$) located in four districts of Punjab (Jhang: 31° 26′ 8″N, 72° 32′ 35″E; Qasur: 31° 12′ 21″N, 74° 45′ 81″E; Sheikhupura: 31° 72′ 26″N, 73° 98′ 80″E; and Vehari: 30° 1′ 13″N, 72° 35′ 94″E), Pakistan from March to August 2015.

2.2. Blood samples

Based on the type of bovid, breed and time of stay of imported cattle in Pakistan, samples were divided into five groups. Groups 1 ($n = 39$), 2 ($n = 40$) and 3 ($n = 68$) had cattle imported to Pakistan from Australia which remained in the country for one, two and three years, respectively; whereas, groups 4 ($n = 50$) and 5 ($n = 49$) contained cross-bred *Bos indicus* (Sahiwal) and *Bos taurus* (Holstein and Friesian) cattle, and buffaloes, respectively. Blood samples were collected from the jugular vein and collected into EDTA-coated tubes and stored at 4 °C until DNA extraction use. The study was conducted according to the animal ethics guidelines and approved by the research committee, College of Veterinary and Animal Sciences, University of Bahawalpur, Pakistan.

2.3. DNA extraction, conventional PCR amplification and DNA sequencing

DNA was extracted from 200 μL of each blood sample using the DNeasy Blood and Tissue Kit (Qiagen, USA), following the manufacturer's instructions and eluted in 100 μL buffer, and were sent to the University of Melbourne for further analysis.

Conventional PCR was carried out to characterise all prevalent genotypes of *T. orientalis*. Primers MPSP-F and MPSP-R were used for the amplification of ~776 bp region of the major piroplasm surface protein (*MPSP*) gene, as described previously (Ota et al., 2009). PCRs were conducted in 25 μL volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM $MgCl_2$, 6.25 μM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, and 1 U of Go*Taq* polymerase (Promega, USA). The PCR cycling protocol was: an initial denaturation at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 5 min. Negative and positive (*T. orientalis*) controls were included in each PCR run (Gebrekidan et al., 2016b). PCR products were examined by agarose (1.5%) gel electrophoresis.

PCR products were then treated with shrimp alkaline phosphatase and exonuclease I (Germentas Inc., USA) using the previous protocol (Werle et al., 1994), and subjected to bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, CA, USA) using (separately) the same primers employed in PCR. The quality of nucleotide sequences was appraised using the program Geneious Pro 5.6.5 (Larkin et al., 2007), and polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes. The *MPSP* sequences were identified by local alignment comparison (six reading frames) using amino acid sequences conceptually translated (using an online tool, http://www.ebi.ac.uk/Tools/st/emboss_transeq/) from reference sequences of *T. orientalis* available from the GenBank database.

Nucleotide sequences were aligned using the MUSCLE V 3.8.31 program (Edgar, 2004) and adjusted manually employing the program Mesquite V 3.03 (Maddison and Maddison, 2008). Based on pairwise comparisons, sequence differences were calculated using the program MEGA 6.0. (Tamura et al., 2013).

2.4. Quantitative PCR

In order to estimate the infection intensities of the two pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis*, each DNA sample was also tested using an established multiplexed tandem PCR (MT-PCR) assay (Perera et al., 2015a). Originally, this assay was established for the detection, differentiation and quantitation of the four commonest genotypes of *T. orientalis* (i.e., *ikeda, buffeli*, *chitose* and *type* 5), which infect cattle in Australasia (Perera et al., 2015a). In this study, we used this assay for the two pathogenic genotypes (*chitose* and *ikeda*) of *T. orientalis*. The assay was conducted in the Easy-Plex platform (AusDiagnostics Pty Ltd., Australia), as described previously (Perera et al., 2015a) using primers (AusDiagnostics) designed specifically to the major piroplasm surface protein (*MPSP*) gene (*chitose*) and the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (*ikeda*). Following PCR, the peak high resolution melting (HRM) temperature of each amplicon was compared with those of pre-determined reference temperatures representing each genotype *chitose* (82.1 ± 1.5 °C) and *ikeda* (87.4 ± 1.5 °C) (Perera et al., 2015a). The relative intensity of infection of each of both pathogenic genotypes was expressed as a DNA copy number (Perera et al., 2015a). All amplicons had the peak melting temperatures within respective reference values; randomly selected amplicons representing each genotype were subjected to single-strand conformation polymorphism (SSCP) analysis and targeted sequencing (Cufos et al., 2012; Perera et al., 2013).

2.5. Phylogenetic analyses

In order to establish the relationship of *MPSP* sequences of *T. orientalis* determined herein and those characterised in previous studies, analyses were performed using Bayesian Inference (BI) and Neighbor Joining (NJ) methods. Included in the analyses were *MPSP* sequences representing *T. orientalis* from cattle from Pakistan and key reference sequences for *T. orientalis* from previous studies (Kawazu et al., 1992; Kubota et al., 1996; Kakuda et al., 1998; Kawazu et al., 1999; Kim et al., 1998; Gubbels et al., 2000; Zakimi et al., 2006; Ota et al., 2009; Yokoyama et al., 2011; Altangerel et al., 2011a, 2011b; Islam et al., 2011; Kamau et al., 2011; Khukhuu et al., 2011; Sivakumar et al., 2013; Bawm et al., 2014; Bogema et al., 2015; Elsify et al., 2015; Jenkins et al., 2015; Gebrekidan et al., 2016b; Pulford et al., 2016; Zhou et al., 2016) from the GenBank database as well as *T. annulata* (outgroup); these sequences were aligned over 596 bp, and the alignment was adjusted manually. BI analysis was conducted using Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criterion (AIC) test in jModeltest v2.1.5 (Darriba et al., 2012). The Tamura 3-parameter model of evolution, with gamma distribution and a proportion of invariable sites (T92 + Γ + I), was utilized for the analysis of the sequence data. The estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100th tree being saved. A consensus tree (50% majority rule) was constructed based upon the remaining trees generated by BI. The NJ analysis was performed using the program MEGA 6.0, and the nodes were tested for robustness with 10,000 bootstrap replicates. The phylogenetic trees produced from the BI and NJ analyses were compared for concordance in topology.

2.6. Statistical analyses

Pearson's chi-square contingency table analysis, Fisher's Exact Test, *t*-test and descriptive statistics were used to analyse the prevalence of *T. orientalis* genotypes detected using conventional and multiplexed tandem PCR assays. The DNA copy number (an estimate of intensity of