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Use of multiplexed tandem PCR to estimate the prevalence and intensity of *Theileria orientalis* infections in cattle

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ABSTRACT

This study employed a semi-quantitative, multiplexed tandem PCR (MT-PCR) to assess the prevalence and infection intensity of four genotypes (*buffeli*, *chitose*, *ikeda* and *type* 5) of *Theileria orientalis* in cattle in Australia. Genomic DNA samples from blood samples ($n = 448$) collected from 27 to 32 dairy cows from each of 15 dairy herds with a history of recent theileriosis outbreaks (Group 1), and from blood samples available from 24 cows with or without oriental theileriosis (Group 2) were tested using MT-PCR. Results revealed that all four genotypes were present in Group 1 cattle; genotype *buffeli* had the highest prevalence (80.5%), followed by genotypes *ikeda* (71.4%), *chitose* (38.6%) and *type* 5 (20.3%). Genotype *ikeda* had the highest average infection intensity in the cattle (relating to 55,277 DNA copies), followed by *buffeli*, *chitose* and *type* 5 (6354–51,648 copies). For Group 2, results indicated that genotype *ikeda* had a significantly higher average intensity of infection than *buffeli* in symptomatic cattle ($P < 0.001$), and symptomatic cattle had a higher intensity of *ikeda* than asymptomatic cattle ($P = 0.004$). Future studies should assess the utility of the present MT-PCR assay as a diagnostic and epidemiological tool in other parts of Australasia and the world.

1. Introduction

Oriental theileriosis of cattle is caused by *Theileria orientalis*, a group of haemoprotistan parasites transmitted by ixodid ticks. This disease is manifested by pyrexia, haemolytic anaemia, productivity losses, abortion and/or mortality (Izzo et al., 2010; Aparna et al., 2011; Islam et al., 2011; McFadden et al., 2011; Perera et al., 2014). To date, 11 distinct genotypes of *T. orientalis* have been characterised using the major piroplasm surface protein (*MPSP*) gene (reviewed by Sivakumar et al., 2014); five of these genotypes (i.e., *buffeli*, *chitose*, *ikeda*, and *types* 4 and 5) have been reported in Australia (Islam et al., 2011; Kamau et al., 2011; Cufos et al., 2012; Perera et al., 2013). Recently, some *T. orientalis* genotypes (including *chitose* and *ikeda*) have been inferred to be involved in significant outbreaks of oriental theileriosis in cattle in the Asia–Pacific region (Izzo et al., 2010; Aparna et al., 2011; Islam et al., 2011; McFadden et al., 2011; Perera et al., 2014).

Traditionally, the diagnosis of oriental theileriosis was based on the detection of piroplasms of *T. orientalis* in blood smears, and/or the use of serological or conventional molecular techniques (Becerra et al., 1983; Kawazu et al., 1992; Tanaka et al., 1993; Kakuda et al., 1998; Jeong et al., 2005; Altay et al., 2008). However, each of these methods has limitations, such as low diagnostic sensitivity and/or specificity. Recently, we established and validated a multiplexed-tandem PCR (MT-PCR) assay (Perera et al., 2015) to overcome these limitations. This method allows the simultaneous detection and differentiation of the four commonest genotypes (i.e., *buffeli*, *chitose*, *ikeda* and *type* 5) of the *T. orientalis* complex in Australasia as well as their semi-quantitation in bovine blood samples. This assay is a cost-effective, practical and specific diagnostic tool whose analytical sensitivity is ~1000 times greater than conventional PCR (Perera et al., 2015), suggesting that it will provide a useful epidemiological tool for rapidly tracking disease outbreaks and for the surveillance of infections. Here, we assessed the prevalence and intensity of infections of four genotypes of *T. orientalis* in dairy cattle in an endemic region in south-eastern Australia, following recent outbreaks of theileriosis. In addition, the infection intensity of pathogenic and apathogenic

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genotypes of *T. orientalis* was assessed using blood samples available from a previous study from symptomatic and asymptomatic cattle suffering from oriental theileriosis.

2. Materials and methods

2.1. Farms, collection of blood samples and haematological examination

For this study, 448 blood samples (Group 1) were collected by registered practicing veterinarians from 27 to 32 cows from each of 15 dairy herds randomly selected in three geographical regions (Bairnsdale, Leongatha and Maffra) of the state of Victoria, Australia, following oriental theileriosis outbreaks between September 2012 and June 2013 (Table 1). Each cow was clinically examined under the supervision or by a registered veterinarian (bovine specialist: J.M.). The clinical signs of theileriosis include pale vulval mucous membranes, anorexia, lethargy and/or increased heart rate (cf. Perera et al., 2014). During the period of blood sampling, there was no clinical evidence of any infectious disease other than oriental theileriosis in any of the herds studied. Individual cattle in these herds were also monitored routinely for mastitis. Information on age and breed of cattle, and the number of clinically affected cattle as well as the number of deaths associated with theileriosis were obtained. In addition, 24 blood samples (Group 2) available from a previous study (Perera et al., 2014) were included to estimate the intensity of infections of *T. orientalis* genotypes; 12 samples were from individual cattle with clinical signs of oriental theileriosis, and 12 from healthy cows with no signs of this disease. For each blood sample, the packed cell volume (PCV) was determined using a micro-haematocrit (Coulter® AcT Diff, Beckman Coulter Inc., USA). Based on PCV values, cattle were classified as normal (“non-anaemic”; >0.24), mildly anaemic (0.15–0.24) or severely anaemic (<0.15).

2.2. Molecular methods

Genomic DNAs were isolated from individual blood samples (200 μl) using the DNeasy blood and tissue kit (cat. No. 69506; Qiagen, USA) and eluted in 100 μl. MT-PCR was conducted using primer pairs designed specifically (by AusDiagnostics Pty Ltd, Australia; cat. No. 38170R) to the piroplasm surface protein (*p23*) gene (genotype *buffeli*), *mpsp* gene (*chitose*), the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (*ikeda*) and *mpsp* gene (*type* 5) in the Easy-Plex platform (AusDiagnostics), precisely as described previously (Perera et al., 2015). Following primary and secondary amplifications, the peak high resolution melting (HRM) temperature of each amplicon was compared with those of predetermined reference temperatures representing individual genotypes: *buffeli* (83.6 ± 1.5 °C), *chitose* (82.1 ± 1.5 °C), *ikeda* (87.4 ± 1.5 °C) and *type* 5 (81.6 ± 1.5 °C) (Perera et al., 2015). The relative intensity of infection of each of these four genotypes was expressed as DNA copy number (Perera et al., 2015). Amplicons with peak melting temperatures that diverged from respective reference values were subjected to single-strand conformation polymorphism analysis (SSCP), and selected samples sequenced (cf. Cufos et al., 2012; Perera et al., 2013).

2.3. Statistical analyses

Analyses were performed on log-transformed data. An independent sample *t*-test was performed to compare the intensity of infection (equating to DNA copies) of each genotype between anaemic and non-anaemic cattle (Group 1), and between symptomatic and asymptomatic cattle (Group 2), using either non-anaemic or asymptomatic cattle group as a reference. A paired-sample *t*-test was used to compare the intensity of infection of dominant genotypes (dominant by prevalence) within each farm, within anaemic and non-anaemic cattle (Group 1) or within symptomatic and asymptomatic cattle (Group 2), using the genotype with the lowest mean DNA copy number as a reference. The SPSS Statistics 22 package (IBM) was used for statistical analyses. A *P*-value of <0.05 was considered as statistically significant.

3. Results

3.1. Prevalence of T. orientalis genotypes

In Group 1, 53.8% (241/448) of cattle were test-positive for *T. orientalis* by MT-PCR (Table 1), and the highest prevalence (63.8%; 95/149) was recorded in the Bairnsdale region, followed by Leongatha (51.7%; 76/147) and Maffra (46.1%; 70/152). Epidemiological data also revealed that the highest number ($n = 36$) of cow deaths had occurred in the Bairnsdale region (Table 1). All four genotypes (i.e., *buffeli*, *chitose*, *ikeda* and *type* 5) were detected in all three geographical regions, and *buffeli* had the highest prevalence (80.5%; 194/241), followed by *ikeda* (71.4%; 172/241), *chitose* (38.6%; 93/241) and *type* 5 (20.3%; 49/241). Fourteen cattle farms in the different regions had the highest prevalence of each genotype *buffeli* and *ikeda*, and one farm (Bairnsdale-4) had *chitose* as the dominant genotype (Table 1). In Group 1, multiple genotypes of *T. orientalis* were more commonly detected in cattle than single genotypes (Fig. 1). Mixed infections with genotypes *buffeli* and *ikeda* had a higher prevalence (32.0%) than any other combination of genotypes (14.1%). For cattle infected with only one genotype, *buffeli* had the highest prevalence (14.1%), followed by *ikeda* (11.2%), *chitose* (7.5%) and *type* 5 (0.8%) (Fig. 1).

3.2. Infection intensities

Although all four genotypes of *T. orientalis* were detected in cattle on all 15 farms (Group 1), the relative intensity of infection with genotype *ikeda* was dominant (with an average DNA copy number of 55,277), followed by *buffeli* (51,648), *chitose* (32,887) and *type* 5 (6354) (Table 1). However, on four farms (i.e., Bairnsdale-1, Bairnsdale-2, Leongatha-5 and Maffra-1) that had experienced severe theileriosis outbreaks (as revealed by the numbers of sick animals and cow deaths prior to the blood collection), the relative intensity of infection with genotype *ikeda* or *chitose* was higher than for *buffeli* (Table 1). Pairwise comparisons of the relative intensity of infection in cattle (on each of the 15 farms) revealed that three of four farms (that had experienced severe theileriosis outbreaks) had a significantly higher intensity of infection with genotype *ikeda* (Bairnsdale-1: $P = 0.026$; Maffra-1: $P = 0.014$) or *chitose* (Leongatha-5; $P = 0.014$) than *buffeli* (Table 2). However, on farm Bairnsdale-5, where there had been 6 cow deaths in late 2012, but no symptomatic cattle at the time of blood collection, the intensity of infection with genotype *buffeli* was higher than for *chitose* ($P < 0.001$) (Table 2).

Based on PCV values (444 bloods samples), cattle in Group 1 were categorised as mildly anaemic (PCV 0.15–0.24; $n = 39$) or “normal” (PCV > 0.24; $n = 405$) (Supplementary Table 1). Overall, the prevalences of genotypes *buffeli* and *ikeda* were high (64.1%) in mildly anaemic animals, and that of genotype *buffeli* was higher (41.7%) in cattle that had normal PCVs (Supplementary Table 1). The inferred intensity of infection with one or more of the four genotypes did not differ significantly between mildly anaemic and non-anaemic cattle (Supplementary Table 2); however, the intensity of infection with the genotype *buffeli* was significantly higher than that with *ikeda* in non-anaemic cattle ($P = 0.010$) (Table 3).