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Original article

Investigating the first outbreak of oriental theileriosis in cattle in South Australia using multiplexed tandem PCR (MT-PCR)

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ABSTRACT

This study investigated the first outbreak of oriental theileriosis in a herd of beef cattle in South Australia using a newly established multiplexed tandem PCR (MT-PCR) to identify, differentiate and quantitate the four genotypes (*buffeli*, *chitose*, *ikeda* and *type* 5) of *Theileria orientalis* recognised to occur in Australasia. Following clinical diagnosis of oriental theileriosis (based on clinical signs, laboratory findings and *post mortem* examination), 155 blood samples were collected from individual cows ($n = 85$) and calves ($n = 70$), and tested by MT-PCR. In total, 117 (75.48%) cattle were shown to be test-positive for *T. orientalis*. All four genotypes were detected, and *ikeda* had the highest prevalence (90.6%; 106/117), followed by *buffeli* (83.8%; 98/117), *chitose* (18.8%; 22/117) and *type* 5 (5.1%; 6/117). Mixed infections with genotypes *buffeli* and *ikeda* had a higher prevalence (55.5%; 65/117) than any other combination of genotypes. The prevalences of *buffeli* and *ikeda* were significantly higher ($P < 0.005$) than those of *chitose* and *type* 5. The average intensity of infection with genotype *ikeda* (329,775 DNA copies) was significantly higher ($P < 0.0001$) than *buffeli* (212,843) and *chitose* (125,462). This study reinforces the utility of MT-PCR as a diagnostic tool for rapidly investigating oriental theileriosis outbreaks in cattle herds and as a pre-movement screening test for preventing the introduction of this disease into non-endemic regions.

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Introduction

Oriental theileriosis is a tick-borne disease of bovids caused by members of the *Theileria orientalis* complex. This disease is transmitted principally by ixodid ticks of the genus *Haemaphysalis* and has a worldwide distribution. Globally, eight distinct genotypes of *T. orientalis* complex, including *type* 1 (*chitose*), *type* 2 (*ikeda*), *type* 3 (*buffeli*) and *types* 4–8, are currently recognised based on the sequence of the major piroplasm surface protein (*MSPS*) gene (reviewed by Sivakumar et al., 2014), five of which have been identified in Australia (Kamau et al., 2011; Perera et al., 2013). Using the same gene, three more genotypes of *T. orientalis*, designated *N*-1, *N*-2 and *N*-3, have been reported to infect sheep, water buffalo and cattle, respectively (Khukhuu et al., 2011). Of these 11 genotypes, only *ikeda* and *chitose* are known to be pathogenic and cause considerable morbidity (including high fever, anaemia, jaundice and abortion), production losses and/or mortality (e.g., Izzo et al., 2010; Islam et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014, 2015a).

Traditionally, in Australia, *Theileria* infection in cattle was reported to be asymptomatic, although some clinical cases had been reported in the state of Queensland in the 1960s (Rogers and Callow, 1966). However, over the last decade hundreds of oriental theileriosis outbreaks have been diagnosed in beef and dairy cattle herds in Australia (e.g., Izzo et al., 2010; Islam et al., 2011; Perera et al., 2013). For instance, Izzo et al. (2010) reported outbreaks to be associated with haemolytic anaemia, jaundice, abortion and mortality in both dairy and beef cattle infected with *T. orientalis* from a subtropical climatic zone (New South Wales, NSW). Islam et al. (2011) reported the first outbreak of oriental theileriosis in a beef herd in a temperate zone of Victoria, Australia. The precise reasons for an increase in the number of theileriosis outbreaks in Australia are unknown.

The most commonly used methods for the diagnosis of theileriosis have been clinical signs, demonstration of *Theileria* piroplasms (a parasite stage within erythrocytes) in stained blood smears and/or characteristic *post mortem* findings such as jaundiced carcass with pale liver and kidneys (Izzo et al., 2010; Biddle et al., 2013). Recently, a number of studies also used conventional PCR

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employing the *MPSP* as a marker to establish the genotypes of *T. orientalis* present (Islam et al., 2011; Kamau et al., 2011; McFadden et al., 2011; Eamens et al., 2013). Although useful, some conventional PCR assays for the diagnosis of *T. orientalis* infection have not been able to identify or differentiate genotypes and estimate infection intensity. To circumvent these limitations, we recently developed a multiplexed tandem PCR (MT-PCR) that allows the simultaneous detection, differentiation and semi-quantitation of four common genotypes (*buffeli*, *chitose*, *ikeda* and *type* 5) of *T. orientalis* (see Perera et al., 2015b). We showed the utility of this MT-PCR assay to rapidly estimate both prevalence and intensity of *T. orientalis* genotypic infections following outbreaks in Australia (Perera et al., 2015c) and New Zealand (Perera et al., 2015d). This assay has a high diagnostic specificity (97.0%) and sensitivity (98.8%) and has proved to be cost effective and practical as a laboratory-based assay (Perera et al., 2015b); it achieves semi-quantitative genotypic diagnosis, has an analytical sensitivity that is ~1000 times greater than conventional PCR and can detect 0.25 parasites per microliter of blood (Perera et al., 2015b). Here, we employed this MT-PCR to study the first outbreak of oriental theileriosis in a herd of beef cattle in South Australia (SA).

Materials and methods

Case history

On 22nd March 2014, a beef cattle farmer introduced 54 Black Angus steers (12–14 months old) from Hernani, NSW (−30°16′44″ S, 152°23′49″ E), an area known to be endemic for oriental theileriosis, into his herd located in Furner, SA (−37°24′22.3″ S 140°20′21.4″ E). Following agistment on paddock A for three days, the steers were transferred to paddock B on the same farm, where they remained for almost two months. On 23rd May, these steers were moved to paddock C on the same farm and 85 Black Angus cows aged between 2 and 9 years, already present on the farm, were moved into paddock B. Following calving in August 2014, one cow died on 4th October 2014. Subsequently, over a period of one week, 10 cows showed cardinal clinical signs of symptomatic oriental theileriosis, including anaemia, pale mucous membranes and depression (Izzo et al., 2010; Islam et al., 2011; McFadden et al., 2011; Perera et al., 2013, 2014), and three of them died.

Clinical diagnosis

A registered veterinarian investigated the outbreak and collected blood samples from five sick cows for haematological examination, which revealed that two cows were test-positive for *Theileria* piroplasms by microscopic examination of Giemsa-stained blood smears, and same animals had a low erythrocyte count (mean: 1.66; normal range: 5.00–10.00 × 10^{12}/l), haemoglobin (mean: 51; normal range: 80–150 g/l) and packed cell volume (mean: 0.14; normal range: 0.24–0.46). *Post mortem* examination of two cows revealed pale and enlarged liver, kidneys and adrenal glands, and mild, diffuse interstitial oedema in the heart and lungs. Based on clinical examination, laboratory diagnosis, characteristic *post mortem* findings and a lack of evidence of any other cause of anaemia and/or abortion (e.g., leptospirosis, babesiosis, post-parturient hypophosphatemia; lantana, fireweed, bracken fern or copper poisoning; Izzo et al., 2010), the clinical diagnosis of oriental theileriosis was made.

Molecular diagnosis by MT-PCR

As the microscopic examination of blood smears does not allow the differentiation of pathogenic from apathogenic *T. orientalis*, we molecularly tested individual blood samples from all cattle in the herd in order to detect, differentiate and quantitate four common genotypes of *T. orientalis*. For this testing, blood samples ($n = 155$) were collected in December 2014 from beef cattle from the coccygeal vein (using an 18 gauge needle) into EDTA tubes by registered, practicing veterinarians (Sa.Mc. and Se.Mc.). All cattle were of the Black Angus breed, and 54.8% were cows aged between two and nine years, while 45.2% were calves of approximately six to eight weeks of age (Table 1).

Genomic DNAs were extracted from individual blood samples (200 μL) using the DNeasy Blood and Tissue Kit (Cat. no. 69,506, Qiagen, USA), following the manufacturer's protocol, and eluted in 100 μL buffer, and then tested by MT-PCR. This assay was conducted in the Easy-Plex platform (AusDiagnostics Pty Ltd., Australia) as described previously by Perera et al. (2015b) using primers (Cat. no. 38170R; AusDiagnostics) designed specifically 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). Following primary and secondary amplifications, the peak high resolution melting (HRM) temperature of each amplicon was compared with those of pre-determined 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., 2015b). The relative intensity of infection of each of these four genotypes was expressed as a DNA copy number (Perera et al., 2015b). 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).

Statistical analyses

Biographical details (ear tag numbers, age and calving date) and details of laboratory examinations for each of 155 study animals were entered into a Microsoft Excel 2010 spreadsheet. DNA copy numbers (a measure of the intensity of infection) for the four *T. orientalis* genotypes for each cow were log-transformed ($\log_{10}$) prior to analysis. DNA copy numbers for each genotype were analysed by one-way analysis of variance and differences among group pairs evaluated using Tukey's Honestly Significant Difference test. Differences in the prevalence of infection across the four genotype groups was assessed using Pearson's chi-squared test. Differences in prevalence among group pairs was assessed using Pearson's chi-squared test with a Bonferroni correction. The SPSS Statistics 21 package (IBM) was used for statistical analyses, and a *P*-value of <0.05 was considered as statistically significant.

Results

A total of 117 of the 155 (75.5%) cattle were test-positive for *T. orientalis* by MT-PCR (Table 1). Although the overall prevalence of *T. orientalis* was higher in cows (85.9%; 73/85) than calves (62.8; 44/70), this difference was not statistically significant. Three to four year-old cows had the highest prevalence (93.7%; 15/16) of *T. orientalis* infection, followed by older cows (up to nine years old) (85.4% 41/48), two year-old cows (80.9%; 17/21), calves from three to four year-old cows (73.3%; 11/15) and calves from four to nine year-old cows (60.0%; 33/55) (Table 1).

All four genotypes (i.e., *buffeli*, *chitose*, *ikeda* and *type* 5) were detected, and *ikeda* had the highest prevalence (90.6%; 106/117), followed by *buffeli* (83.8%; 98/117), *chitose* (18.8%; 22/117) and *type* 5 (5.1%; 6/117). Genotypes *ikeda* and *buffeli* were detected in cattle of all age groups, but genotypes *chitose* and *type* 5 were not detected

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