Veterinary Parasitology 245 (2017) 29–33

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Monitoring *Theileria orientalis* (Ikeda)-associated bovine anaemia in affected cattle over time

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ARTICLE INFO

Keywords:
Theileriosis
Cattle
Bovine
Theileria orientalis
Ikeda
Surveillance
Epidemic
Regenerative anaemia
Theileria-associated bovine anaemia

ABSTRACT

The aim of the study was to observe changes in haematocrit (HCT) over time in a New Zealand South Island dairy herd affected by an outbreak of *Theileria*-associated bovine anaemia (TABA; Ikeda). A secondary aim was to relate individual cow HCTs to the amount of *Theileria orientalis* Ikeda DNA present in the blood, as measured by cycle threshold values, using a quantitative PCR (qPCR).

Over a 6 month period, blood samples from 19 randomly selected cattle were monitored from a herd of 600 dairy cows. The sampling interval was approximately fortnightly for the first six weeks, followed by sampling at between four and six week intervals.

At the initial report of the outbreak, two from six cattle were anaemic (HCT < 0.25 L/L). Blood collected from 14 cattle 11 days later showed that 57% (95% CI 33–77%) of the cattle sampled were anaemic. Of the 19 cattle that went on to be monitored, 12 (63% 95% CI = 41–81%) developed anaemia at some point during the period of monitoring. One of the anaemic animals did not meet the case definition for TABA Ikeda. For individual cattle, the average number of days between when cattle were first detected as anaemic and when HCT returned to normal was 53 days (median = 47 days, range = 6–92 days). At the point of notification the amount of *T. orientalis* Ikeda DNA in the blood of the six cattle tested was low (Cq median = 36), but 11 days later the amount of DNA in blood of 14 additional cows tested was relatively high (Cq median = 24). Levels of all 19 cows monitored continued to remain moderately high through the period of testing (Cq median = 29). This was despite a general improvement in the HCT of affected cattle.

In four of the 15 cattle positive to *T. orientalis* Ikeda where blood fractions (plasma and whole blood) were tested, it appeared that *T. orientalis* Ikeda (as measured by qPCR) dropped more rapidly in plasma fractions than in whole blood at the point that HCT started to return to normal levels.

Despite the assumption that tick populations were low in the Canterbury region of the South Island the impact of TABA (proportion of herd affected and the average period that animals remained anaemic) on the case herd was still relatively high.

1. Introduction

Theileria orientalis is a blood-borne parasite with worldwide distribution that was first detected in New Zealand in 1983 (James et al., 1984). It is transmitted by *Haemaphysalis longicornis*, the only livestock tick established in New Zealand (Heath 2016). Different genotypes of *T. orientalis* have been identified using the major piroplasm surface protein (MPSP) p32 gene (Khukhuu et al., 2011; Perera et al., 2013). The four main MPSP genotypes are type 1 (Chitose), 2 (Ikeda), 3 (Buffeli) and p32 (type 5). All four types have been detected in New Zealand (Pulford et al., 2016a). *Theileria orientalis* Chitose and Ikeda have both been associated with clinical disease in Australia, Japan and New Zealand (Watts et al., 2016).

In late 2012 outbreaks of TABA were reported in dairy and beef-cattle herds in New Zealand (McFadden et al., 2013; Lawrence et al., 2013). Strain typing of *T. orientalis* was carried out on blood samples collected from cattle herds experiencing outbreaks. One of the strains identified was *T. orientalis* Ikeda type. Analysis of data from the New Zealand outbreaks showed that there was a greater likelihood of *T. orientalis* Ikeda type being present in cattle from herds experiencing

Abbreviations: AHL, Animal Health Laboratory; Cq, quantification cycle threshold values; HCT, Haematocrit; MPI, Ministry for Primary Industries; MPSP, Major Piroplasm Surface Protein; rbc, Red blood cells; T. orientalis, *Theileria orientalis*; TABA, *Theileria*-associated bovine anaemia; qPCR, quantitative PCR

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http://dx.doi.org/10.1016/j.vetpar.2017.07.035
Received 30 January 2017; Received in revised form 10 July 2017; Accepted 31 July 2017

outbreaks of anaemia compared to non-outbreak herds. In addition, individual animals within an affected herd were more likely to be anaemic if the Ikeda type was present compared with animals with endemic strains of *T. orientalis* such as the Chitose type (McFadden et al., 2013; Lawrence et al., 2013). Outbreaks of TABA (Ikeda) can be subtle, often with only a small proportion of the herd detected with obvious clinical signs; however, where laboratory analysis is carried out on a sample of the herd, a high prevalence of anaemia maybe detected (McFadden et al., 2013; Pulford et al., 2016a). Prior to *T. orientalis* Ikeda being detected in cattle in New Zealand, anaemia was considered to be a relatively uncommon event.

Since the initial diagnosis of TABA (Ikeda) the Ministry for Primary Industries (MPI) has continued to monitor the New Zealand epidemic through various surveillance initiatives. These have included case reporting (with subsidised PCR testing), syndromic surveillance, sentinel surveillance, serological surveillance as well as specific active surveillance initiatives to understand the tick vector distribution (McFadden et al., 2016a). Data from these activities have shown that the number of affected cattle herds has continued to increase over time with seasonal peaks in autumn and spring coinciding with peak activity of nymph and adult ticks. In spring 2014, the epidemic extended into areas of the South Island that were previously considered to be of low tick risk. As a result a survey of the tick vector was initiated that showed that ticks were present in areas outside of the known distribution (McFadden et al., 2016a).

More intensive monitoring has been carried out on individual farms experiencing an outbreak of TABA (McFadden et al., 2013; McFadden et al., 2015; Pulford et al., 2016a). This form of monitoring has been important to inform on risk, and understand the epidemiology and impact of the disease. We describe results from monitoring individual cattle during an outbreak of TABA (Ikeda) on a dairy farm in the Canterbury region of the South Island of New Zealand.

2. Materials and methods

2.1. Initial investigation and monitoring

The Ministry for Primary Industries was notified of an outbreak of TABA (Ikeda) in a South Island dairy farm on 26 November 2015. The affected herd consisted of 600 Jersey-Friesian cross milking cows. The field veterinarian making the notification had randomly selected and tested six cows from the herd to determine if TABA was present. Two of these cattle had haematological signs of anaemia (2/6; 33%); where cattle were classified as anaemic if HCT < 0.25L/L (Riond et al., 2008). Anaemic cattle were positive for *T. orientalis* Ikeda using PCR thus fitting the case definition for TABA (Ikeda) described by McFadden et al. (2013).

MPI advised the veterinarian that a further 14 animals be sampled to gain a greater confidence in the prevalence of TABA occurring in the herd at the time. Other priorities with both the servicing veterinarian and the farmer meant that the additional 14 cattle were not sampled until 11 days later. The additional cattle were randomly selected for sampling by the veterinarian as they entered the milking parlour.

Over the course of 6 months, additional blood samples from the combined group of cattle were collected on a number of other occasions. One of the 20 cattle from the initial sampling group left the herd and became unavailable for further sampling. Thus the total number of cattle subsequently monitored was 19. The farm was a commercial entity and the regime of samplings was dictated by both farmer and veterinary constraints. For the first 6 weeks of monitoring, collection took place at fortnightly intervals. Subsequently this interval was extended to between 4 and 6 weeks as there had not been a dramatic rebound in HCT as had been expected. On some occasions animals from the monitored group were not available to be bled, but were sampled at subsequent collection dates. The aim of monitoring the group was to observe the change in HCT over time; but also at the individual cow level to determine the amount of *T. orientalis* Ikeda DNA as measured by qPCR and relate this to the HCT.

2.2. Molecular testing

A full description of the molecular techniques to detect *T. orientalis* Ikeda and Chitose is given in Gias et al. (2016) and Pulford et al. (2016b). In addition fractionated whole blood in EDTA was prepared by centrifugation (2,500x*g* 10mins) and the plasma fraction was removed. Whole blood and plasma fractions were diluted 1:5 in sterile water prior to DNA extraction. Samples were processed on a Corbett Extractorgene robot to extract DNA from samples and were eluted in 200ul of diethylpyrocarbonate (DEPC) treated sterile water. Relative PCR efficiency for each sample extract was evaluated for all samples by comparison of 18S gene amplification. Ikeda genomes were measured in samples using a *T. orientalis* Ikeda specific TaqMan qPCR for the precise identification of Ikeda MPSP gene (Pulford et al., 2016b).

Cycle threshold values (Cq) from qPCR were recorded and the inverse values calculated as 1/qPCR cycle threshold * 100 for the purpose of charting quantitative gene copy data. Thus a value of 3 on the inverse Cq scale was equivalent to a Cq of 33 indicating low Ikeda MPSP gene copies (low Cq range 31–38), a value of 4 was equivalent to Cq = 25 (medium gene copy Cq range 25–30); and a value of 5 was equivalent to Cq = 20 (high gene copy Cq range 18–24). An inverse qPCR value below 2.6 (Cq = 38) was considered to be negative. The cycle threshold value arbitrarily assigned where no MPSP gene copies were detected was 45, corresponding to an inverse Cq value of 2.2.

The molecular technique used to detect the bovine haemoplasmas is described in McFadden et al. (2016b).

2.3. Statistical analysis

Graphical presentation of data and confidence intervals were carried out using the ggplot2 and epiR packages, respectively in R v3.2.2 (R Development Core Team, 2011; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

At the time of the report of the outbreak to MPI, the cattle herd was checked for evidence of ticks; however, none were detected by the herd manager and farm veterinarian. A single tick specimen, found on the farm dog used to help manage cattle, was confirmed as *Haemaphysalis longicornis* by a MPI entomologist. This find confirmed that ticks were present in the farm environment, though not the location on the farm or the level of environmental infestation. There was also a more recent record of cattle ticks from horses in close vicinity to the outbreak property (ACG Heath, pers. com) confirming that the ecology in the general location was likely to be suitable for tick inhabitation.

Eleven days after the initial report of two from six cows with anaemia, an additional 14 cows were sampled. At this sampling 57% (95% CI 33- 77%) of the cattle sampled were anaemic. Thus a total 20 cattle were tested during this initial round of sampling. Of these 20 cattle, 19 cattle (a combination of the six initially reported and 13 from 14 tested above) went on to be monitored further. Of the 19 cattle monitored, 12 (63% 95% CI = 41–81%) developed anaemia at some point during the period of monitoring. One of those animals that became anaemic for a brief period (24 days, lowest HCT = 0.24 L/L) was negative for *T. orientalis* Ikeda on all occasions tested (Fig. 2, id = 931).

Monitoring showed that the HCT for all cattle tested had returned to normal by day 186 (6 months) on the 23 May 2016, the day of last sampling (HCT mean = 0.31 L/L, range = 0.28–0.37 L/L), (Fig. 1). For individual cattle, the average number of days between when cattle were first detected as anaemic and when HCT returned to normal (using the end date for anaemia as the mid-point of the date that HCT returned to normal and date of sampling immediately prior to this one) was 53 days