Veterinary Microbiology 219 (2018) 53–62

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Immunopathological changes and apparent recovery from infection revealed in cattle in an experimental model of Johne's disease using a lyophilised culture of *Mycobacterium avium* subspecies *paratuberculosis*

Douglas J. Begg, Karren M. Plain, Kumudika de Silva, Ratna Gurung[1], Alison Gunn, Auriol C. Purdie, Richard J. Whittington*

Farm Animal Health, Sydney School of Veterinary Science, Faculty of Science, University of Sydney, Camden, Australia

ARTICLE INFO

Keywords:
Paratuberculosis
Johne's disease
Cattle
Experimental infection
Pathogenesis
Immune response

ABSTRACT

Johne's disease (JD) or paratuberculosis is an economically significant, chronic enteropathy of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Experimental models of JD in cattle are logistically challenging due to the need for long term monitoring, because the clinical disease can take years to manifest. Three trials were undertaken, the largest involving 20 cattle exposed orally to a low dose of C strain MAP and 10 controls studied for 4.75 years. Frequent blood and faecal sampling was used to monitor immunological and infection parameters, and intestinal biopsies were performed at two time points during the subclinical disease phase. Although clinical disease was not seen, there was evidence of infection in 35% of the animals and at necropsy 10% had histopathological lesions consistent with JD, similar to the proportions expected in naturally infected herds. Faecal shedding occurred in two distinct phases: firstly there was intermittent shedding < ~9 months post-exposure that did not correlate with disease outcomes; secondly, in a smaller cohort of animals, this was followed by more consistent shedding of increasing quantities of MAP, associated with intestinal pathology. There was evidence of regression of histopathological lesions in the ileum of one animal, which therefore had apparently recovered from the disease. Both cattle with histopathological lesions of paratuberculosis at necropsy had low MAP-specific interferon-gamma responses at 4 months post-exposure and later had consistently shed viable MAP; they also had the highest loads of MAP DNA in faeces 4.75 years post-exposure. In a trial using a higher dose of MAP, a higher proportion of cattle developed paratuberculosis. The information derived from these trials provides greater understanding of the changes that occur during the course of paratuberculosis in cattle.

1. Introduction

Johne's disease (JD), or paratuberculosis, is an economically significant, chronic enteropathy of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease affects a range of species, notably cattle, sheep, deer and goats, and is difficult to control due to the chronicity of disease, challenges with diagnosis of infected animals, the lack of treatment options and in some contexts the problem of wildlife reservoirs of MAP (Shaughnessy et al., 2013; Whittington et al., 2012).

Experimental infection models for JD are vital for critical study of pathogenesis and immunity (Begg and Whittington, 2008; Hines et al., 2007). However, experimental inoculation of cattle with MAP has resulted in infection rates ranging from 13% to 100% and usually very few animals have developed clinical disease (Lepper et al., 1989; Mortier et al., 2015). This can be attributed to several factors including the age of the cattle when they were inoculated, the dose of inoculum and the duration of the trial. Incubation periods for natural cases of paratuberculosis are very long, in fact ranging from a few years up to 14 years in cattle (Whittington and Sergeant, 2001) and generally are greater than 2 years (Mortier et al., 2013). Higher doses at a young age are more likely to result in a clinical disease outcome than similar doses given when the animal is older (Mortier et al., 2013; Windsor and Whittington, 2010). However, the use of high doses leads to infection outcomes that may not be comparable to natural infection (Mitchell et al., 2015), because disease may develop faster than expected, pathology may be more severe than usual and immune responses may develop sooner (Begg and Whittington, 2008). However, seeking

* Corresponding author at: The University of Sydney, Sydney School of Veterinary Science, 425 Werombi Road Camden, NSW 2570, Australia.
E-mail address: richard.whittington@sydney.edu.au (R.J. Whittington).
[1] Current address: RG Laboratory Service Unit, National Centre for Animal Health, Serbithang, Bhutan.

https://doi.org/10.1016/j.vetmic.2018.03.029
Received 31 December 2017; Received in revised form 12 March 2018; Accepted 29 March 2018

relevant disease outcomes over a timeframe that is reflective of natural disease development can be prohibitively expensive, as it requires holding large experimental animals for long periods of time under stringent biocontainment and ethical conditions.

As a result, experimental inoculation of MAP in cattle has typically involved small group sizes (approximately n = 4) per treatment, for example in the trials of Allen et al. (2011), Mortier et al. (2013) and Stabel et al. (2009), with most researchers choosing trial end points based on the detection of subclinical infection (Stabel et al., 2009; Sweeney et al., 2009). The use of small numbers of animals may not allow for adequate representation of the spectrum of complex disease outcomes that may be observed in an infected herd: clinical disease, various forms of pathology (multibacillary and paucibacillary), divergent immunological responses (Th1, Th2), slowly progressing subclinical infection, arrested pathogenesis, or even recovery (Begg et al., 2011; Dennis et al., 2011; Whittington et al., 2017).

Longitudinal studies of the immune response over the course of paratuberculosis are important in understanding the pathogenesis of the disease and the diagnostic potential of conventional tests and putative biomarkers. In an experimental model of paratuberculosis in sheep, early (4 months post-exposure) increases in MAP DNA shed in the faeces and reduced MAP-specific interferon-gamma (IFN-γ) responses were predictive of animals that became infectious and developed severe disease (de Silva et al., 2013). In a study of naturally exposed cattle and using a modelling approach, Magombedze et al. (2017) identified that the IFN-γ assay cannot reliably predict infection outcome but faecal shedding can. There is a need for more longitudinal studies in cattle that follow individual animals from the time of exposure and regularly throughout the course of disease, through to defined end points, applying a range of existing and novel, immune and non-immune diagnostic tests.

In prior work, we developed a reproducible experimental infection model for paratuberculosis in sheep (Begg et al., 2010). The aims of this study were to adapt this model for cattle, beginning with a pilot study to confirm the infectivity of a defined cattle (C) strain of MAP, followed by a long term experiment to evaluate the outcomes of low dose infection, simulating natural exposure in non-intensively managed, grazing cattle. A third trial was conducted in which a higher dose of MAP was employed.

2. Methods

2.1. Experimental animals

Fifty neutered male Holstein calves aged 2–4 months were used in 3 trials. The calves were sourced from a dairy farm in Camden, NSW, Australia, which was shown to be free of Johne's disease by on-farm monitoring of the infection status of their dams using antibody ELISA, faecal culture and a direct faecal PCR (Plain et al., 2014) on the whole herd (n = 433) in 2008. All calves were shown to be free from MAP infection by faecal culture, antibody ELISA and IFN-γ ELISA prior to the study. The animals were managed under conventional Australian farming conditions by grazing in open paddocks on unimproved pasture, with handfeeding of lucerne chaff and pellets as required.

2.2. Challenge strain and preparation of the seedstock culture inoculum

Following a similar method to Begg et al. (2010), a C strain of MAP CM00/416, a culture at final passage level 4 (including its primary isolation from cattle faeces), was used as the inoculum. CM00/416 is genomically similar to K10 (Marsh et al., 2006) and is an IS*1311* C strain, IS*900* RFLP type C3 (Marsh and Whittington, 2007). It was reconstituted from lyophilised seed stock and inoculated into a liquid culture medium, either BACTEC 12B (BD) supplemented with egg yolk and mycobactin J (MJ) or M7H9C (Whittington et al., 1999; Whittington et al., 2013). After culture in the liquid medium for 3 weeks at 37 °C, the vial was subcultured onto modified Middlebrook 7H10 medium with MJ and incubated for 4 weeks at 37 °C. The slopes were harvested and single cell suspensions were prepared in phosphate buffered saline (PBS) with 0.1% v/v Tween 20 by repeat mixing on a vortex mixer and by passing the suspension through a 26 gauge needle. Enumeration was by visual counting in a Thoma-ruled counting chamber and by end point titration in a liquid culture medium (BACTEC or M7H9C), using the standard three tube most probable number (MPN) method (Reddacliff et al., 2003; Whittington et al., 2000). The visual counts were used to prepare suspensions at 10^9 MAP/mL for trials 1 and 2, and at 10^{10} MAP/mL for trial 3. Viable counts were calculated retrospectively from the MPN results. One millilitre aliquots of this stock suspension were diluted to 10 mL final volume using PBS, being the amount inoculated in each calf each time. The remaining undiluted suspension (stock) was retained at 4 °C in order to repeat the inoculation one week later, when another MPN enumeration was undertaken. IS*900* and IS*1311* PCR restriction endonuclease analysis (REA) assays were conducted on the stock solution to confirm that the infectious material was MAP C strain (Marsh et al., 1999; Whittington et al., 1998). A fresh suspension was prepared from lyophilised stock for the third inoculation dose.

2.3. Experiments

The number of animals in each experiment and the treatment groups are shown in Table 1. Post-exposure times refer to time elapsed since the date of first inoculation of MAP.

2.3.1. Trial 1

Five calves aged 3 months of age were each inoculated orally with 8.6×10^9 viable MAP cells followed one week later by 1.86×10^8 viable MAP cells. One month after the first inoculation, 8.6×10^8 viable cells were given to each calf. Two of the animals were necropsied two months after the first inoculation; the remaining 3 animals were necropsied 3 months after the initial inoculation.

Table 1
Faecal culture, tissue culture and histopathology results of the cattle in three infection trials.

Trial	No. of animals	No. inoculated with MAP	Time of necropsy[a]	Faecal culture positive[b]	Gross lesions	Histo-pathogical lesions	Culture of tissues	
							Any tissue	Liver or hepatic lymph node[c]
1	2	2	2 mth	0/2	0/2	0/2	2/2	0/2
1	3	3	3 mth	1/3	0/3	0/3	2/3	0/3
2	30	20	4.75 yr	2/20	1/20	2/20	7/20	2/20
3	15	10	9 mth	0/0	0/10	7/10	9/10	0/10

[a] time post-exposure.
[b] at the final sampling timepoint.
[c] these animals were also culture positive from intestinal tissues.