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Research article

Culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from blood and extra-intestinal tissues in experimentally infected sheep

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease or paratuberculosis, a chronic enteritis of ruminants, and has been suggested to play a role in Crohn's disease in humans. While Johne's disease is primarily expressed in the gastrointestinal tract, isolation of MAP from extra-intestinal tissues indicates that microbial dissemination via the haematogenous route may occur during the infection. Consequently, the occurrence of mycobacteraemia and dissemination to the liver and hepatic lymph node was investigated in 111 sheep. Disseminated infection was detected in 18 of the 53 sheep that were confirmed to be infected following oral exposure to MAP while the bacterium was isolated from the blood of only 4 of these animals. Disseminated infection was detected more frequently from animals with a positive compared to a negative faecal culture result, multibacillary compared to paucibacillary lesions, and clinical compared to subclinical disease. Detection of MAP in blood by culture was significantly associated with increased time post-exposure and clinical disease, with trends for increased detection in animals with multibacillary lesions and positive faecal culture results. Isolation of MAP from blood was difficult in the early stages of the disease and in paucibacillary animals as the bacteraemia may be intermittent, below the limit of detection or MAP may be present in a dormant non-culturable form. Prolonged incubation periods prior to growth in BACTEC were consistent with inhibition of growth or dormancy in some blood cultures.

1. Introduction

Paratuberculosis, or Johne's disease, is a widespread disease of both domestic and wild ruminants caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Infection results in a chronic enteritis with a long subclinical phase during which shedding of bacteria in faeces may be present. Two forms of paratuberculosis are widely recognised, paucibacillary and multibacillary (Clarke, 1997). Most clinical cases have multibacillary lesions, in which macrophages in tissue infiltrates are laden with acid fast bacilli. Once clinical signs occur they include diarrhoea in some species, emaciation and death (Clarke, 1997). While the gastrointestinal tract is the primary site of infection and pathology, dissemination of MAP to extra-intestinal tissues occurs. Disseminated infection has been detected by culture of MAP from extra-intestinal tissues from both clinically and subclinically infected cattle with varying serological, gross pathological and histological classifications (Antognoli et al., 2008; Dennis et al., 2008). MAP has also been demonstrated in, or cultured from, liver, reproductive tissues and skeletal muscle of clinically and subclinically affected cattle and sheep (Alonso-Hearn et al., 2009; Ayele et al., 2004; Buergelt et al., 2004; Eppleston and Whittington, 2001; Gwozdz et al., 1997, 2000; Lambeth et al., 2004; Reddacliff et al., 2010). Disseminated infection has also been reported in deer, elk and goats

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(Morin, 1982; Robinson et al., 2008; van Kooten et al., 2006; Williams et al., 1983).

Spread of MAP from the gastrointestinal mucosa may result from haematogenous spread, for example to the liver via the portal circulation, however, there are few reports of the culture of MAP from blood, with one clinically affected dairy cow (Koenig et al., 1993) and two infected sheep reported as blood culture positive (Bower et al., 2010). In contrast to the paucity of culture data, PCR to detect the IS*900* element of MAP from processed blood samples has been applied in many studies. The results indicate great variation in the prevalence and the stage of infection associated with IS*900* positive status. Testing of blood produced a positive PCR result in 66–72% of clinically affected sheep compared with 22–33% of subclinically affected sheep (Buergelt and Williams, 2004; Gwozdz et al., 1997, 2000). Blood samples from subclinically affected cattle and clinically and subclinically affected goats were also PCR-positive (Barrington et al., 2003; Singh et al., 2010). Positive PCR results were detected in ELISA negative and ELISA positive cattle and sheep of varied ages including young animals (Bhide et al., 2006; Juste et al., 2005).

Detection of MAP DNA from human clinical samples is increasingly being attempted due to the proposed link between Crohn's disease (CD) and MAP. Using blood samples, there was considerable variation in the percentage of PCR-positive results that were obtained from patients with CD (16–46%) and healthy controls (20–47%) (Juste et al., 2008; Naser et al., 2004).

In summary, although positive IS*900* PCR results from blood samples have been observed from paratuberculous animals, as well as from humans, the prevalence of bacteraemia detectable by culture and the stages of disease when it occurs, are not yet known. The aim of this study was to determine the occurrence of MAP bacteraemia, by culturing samples collected sequentially from experimentally inoculated sheep.

2. Materials and methods

2.1. Animals and experimental inoculation

All animal experiments in this study were approved by the University of Sydney Animal Ethics Committee. All animals were managed under conventional Australian sheep farming conditions by grazing in open paddocks on unimproved pasture.

Merino or Merino-cross lambs ($n = 111$) aged 3–4 months were used in 4 experiments corresponding to trials 3–6 described by Begg et al. (2010). The lambs were sourced from farms where sheep were shown to be free of Johne's disease by repeated testing of their dams using antibody ELISA, faecal culture of the whole flock and tissue culture of cull sheep. All lambs gave negative results from samples collected prior to the study and examined using faecal culture, antibody ELISA and IFN-γ ELISA tests.

MAP strain Telford 9.2, a clonal culture at passage level 5, isolated from sheep faeces and characterised as IS*1311* S strain, IS*900* RFLP type S1 (Marsh and Whittington, 2007) was used as the inoculum for trials 3–5 and for 20 animals from trial 6. In trial 6, a further 20 animals were inoculated with an intestinal tissue homogenate. The intestinal tissue was obtained from an animal with multibacillary lesions that was confirmed to be infected by the S strain of MAP using IS*900* PCR and IS*1311* PCR REA.

Animals were orally inoculated with 2.3×10^5–2.4×10^8 viable MAP 3 times at the commencement of the study, and animals from trial 3 were given a further 2 inoculations at 11 and 12 months as shown in Fig. 1. Blood and faeces were collected from the experimentally infected animals monthly, three monthly or six monthly as shown in Fig. 1.

The animals were assessed visually for weight loss each week in conjunction with weighing. Animals were defined to have clinical disease if they exhibited severe weight loss determined by 10% body weight loss, and had pathological findings consistent with Johne's disease.

2.2. Culture of blood samples

Prior to blood collection the wool was clipped and the skin was decontaminated with 70% (v/v) ethanol. Blood was collected in 9 ml lithium heparin vacuum blood tubes (Vacutainer, Greiner Bio-one) from the jugular vein using an 18-gauge needle.

Blood samples were cultured using an optimised method (Bower et al., 2010). Briefly, the blood sample was centrifuged at $1455 \times g$ for 20 min with slow brake, and the leukocyte layer (buffy coat) was collected using a sterile disposable polypropylene bulb pipette, combined with 5 ml erythrocyte lysis buffer (0.83%, w/v NH_4Cl, 0.1%, w/v $KHCO_3$, 0.01 M EDTA pH 7.5) and incubated at room temperature for 5 min to allow lysis of the erythrocytes. A 5 ml volume of sterile PBS was added, mixed by inversion

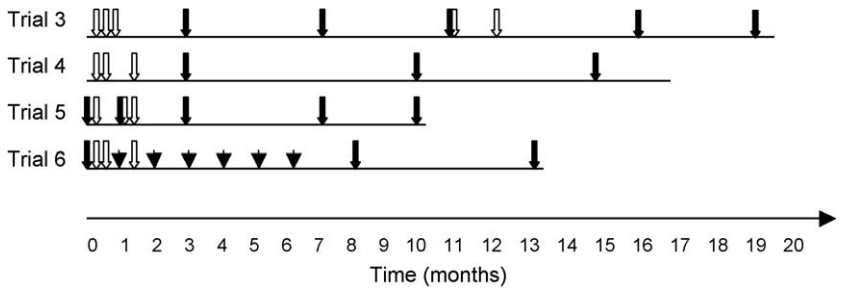

Fig. 1. Experimental infection trials showing the timing of: inoculations, (unshaded arrow); blood and faecal sampling, (solid arrow); blood sampling only (small arrow). Trial number corresponds to Begg et al. (2010).