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Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model

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Abstract

The objective of this study was to develop a short-term experimental infection model for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cattle, using small oral doses of organisms. Specifically, the effect of dose size was evaluated, as well as specific tissue predilection sites for recovery of MAP. Oral doses as low as 1.5×10^6 CFU reliably produced infection that could be detected 3 weeks following infection. Detection of infection required culture of multiple intestinal samples (jejunum and ileum) for MAP. Histological examination did not permit detection at this early stage. Results from this study suggest intestinal mucosa, rather than tonsil, as the primary portal of entry for MAP. The experimental infection model described here is useful for studying the early effects of preventive and therapeutic interventions for paratuberculosis in cattle.

Keywords: Paratuberculosis; Cattle; Johne's disease; *Mycobacterium avium* subsp. *Paratuberculosis,* culture; Histopathology

1. Introduction

Paratuberculosis (Johne's disease) is a costly infectious intestinal disease of cattle and other ruminants with worldwide distribution. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Control of the disease in cattle has been hindered by its long incubation period, the predominance of asymptomatic infected animals that shed the organism in faeces, difficulty in detection of subclinically infected animals, and the continued susceptibility of vaccinated cattle to become infected with and shed MAP organisms.

Numerous studies of bovine experimental infections have been reported. In many of the early studies, the apparent goal was to produce a model that resulted in clinical signs as early as possible. Thus, many of the early reports involved large oral doses (Rankin, 1959; Payne and Rankin, 1961a,b), or intravenous doses (Larsen et al., 1977), that may not have been representative of the typical clinical situation. Experiments conducted by Payne and Rankin, using a large single oral dose (200 mg wet weight) suggested that the tonsils were the primary portal of entry following oral inoculation, and

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the intestinal lesions developed later. However, experiments conducted by Gilmour et al. (1965), using 10 weekly smaller doses showed the small intestine to be the most likely portal of entry, with no evidence for entry of infection via the tonsils. Waters et al. (2003) showed that direct instillation of MAP into the tonsillar crypts of calves resulted in tonsillar and intestinal colonization with MAP.

More recently, attention has focused on the role of M cells in the uptake of MAP in ileal Peyer's patches. Following direct inoculation of MAP into surgically prepared loops of ileum, organisms crossed intestinal epithelium by M cell uptake, (Momotani et al., 1988), suggesting that this might also be the case following oral inoculation. It has even been suggested that the respiratory tract could be the route of infection in cattle, although this has not been tested experimentally (Corner et al., 2004).

Experimental infection models provide useful information about the pathogenesis of paratuberculosis, such as portal of entry, tissue predilection sites, and host immune responses to MAP. Experimental infections may also be used to evaluate the efficacy of interventions, such as immunotherapy or chemotherapy, to prevent or blunt the effect of MAP infection in calves. The objective of this study was to develop an experimental infection model using a smaller, potentially more clinically relevant dose of MAP, and to measure the extent of infection at a short interval following infection to facilitate more rapid results for intervention studies. In order to accomplish this, calves were infected with varying doses of MAP, and MAP culture from multiple tissue sites was implemented to permit early detection of infection status. The results of these cultures also provide additional information about predilection sites for MAP recovery and possible portal of entry following oral infection in neonatal calves.

2. Materials and methods

2.1. Animals

Newborn Holstein calves ($n = 26$) were obtained from a dairy free of paratuberculosis. Each calf was separated from its dam at birth, and fed 4L colostrum from its own dam in the first 24 h of life, followed by milk replacer (2L twice daily) and calf starter grain (free of antimicrobials) throughout the study. Calves were housed in individual pens in an isolation barn, and bedded on straw. The temperature in the barn was equal to the ambient environmental temperature, except in winter when it was heated to 18 °C. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.2. Inoculum

Colonies of MAP, cultured on Herrold's Egg Yolk Medium (HEYM) from a naturally infected cow (ATCC700533), were inoculated into Middlebrook 7H9 broth supplemented with Mycobacin J (2 mg/L) and oleic acid–dextrose–catalase (OADC, 100 ml/L). This was incubated at 37 °C for three weeks, at which time the media was visibly turbid with an OD at 540 nm of 0.51. Aliquots of this first passage MAP were frozen at −70 °C to be used as seed inoculum for each experiment. The inoculum was confirmed to contain the IS900 insertion element by PCR (Vary et al., 1990). Prior to the start of an experiment, seed inoculum was thawed, added to Middlebrook 7H9 broth with OADC and Mycobactin J, and incubated at 37 °C for 3–4 weeks. At this point, the media was visibly turbid with an OD between 0.40 and 0.50. Media was passed through a 20-gauge needle to disrupt clumps, and diluted with sterile water to an OD of 0.32, which corresponds to a concentration of approximately 10^8 CFU/ml. Serial dilutions of the inoculum were made in Middlebrook 7H9 without additives, incubated on HEYM, and colonies counted after 16 weeks incubation to confirm the final concentration of MAP that was in the inoculum. The inoculum was stored at 4 °C until just prior to inoculation, at which time an appropriate volume necessary to provide the desired dose for a particular experiment was diluted in 40 ml of milk replacer, and drawn into a 60 ml plastic syringe. The syringe was placed in the calf's mouth, and the milk replacer–inoculum mixture slowly administered into the mouth, allowing the calf to voluntarily consume the inoculum while it sucked on the syringe, in hopes that the esophageal groove reflex would enhance movement of the mixture into the abomasum.

2.3. Study design

Experiments were conducted to evaluate the effect of inoculum dose and calf's age at inoculation on the recovery of MAP from tissues.

Experiment 1. Six calves were inoculated with 2.5×10^{10} CFU of MAP on day 2 and day 3 of age. Calves were euthanized by intravenous barbiturate on day 42–44 of age, and a necropsy was performed immediately. At post-mortem, extensive tissue sampling was performed for the detection of MAP organisms by bacteriological culture. A total of 39 tissue samples were collected with separate sterile instruments for each sample, including 12 proximal small intestinal sites (two duodenum, 10 jejunum) and the adjacent mesenteric lymph node, three ileum sites (proximal, middle, distal) and two ileocecal lymph nodes, ileocecal valve, cecum, and spiral colon. The 10 jejunum samples (and adjacent lymph node) were taken at approximately equal inter-