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Evaluation and histological examination of a *Campylobacter fetus* subsp. *venerealis* small animal infection model

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ABSTRACT

Bovine genital campylobacteriosis (BGC), caused by *Campylobacter fetus* subsp. *venerealis*, is associated with production losses in cattle worldwide. This study aimed to develop a reliable BGC guinea pig model to facilitate future studies of pathogenicity, abortion mechanisms and vaccine efficacy. Seven groups of five pregnant guinea pigs (1 control per group) were inoculated with one of three strains via intra-peritoneal (IP) or intra-vaginal routes. Samples were examined using culture, PCR and histology. Abortions ranged from 0% to 100% and re-isolation of causative bacteria from sampled sites varied with strain, dose of bacteria and time to abortion. Histology indicated metritis and placentitis, suggesting that the bacteria induce inflammation, placental detachment and subsequent abortion. Variation of virulence between strains was observed and determined by culture and abortion rates. IP administration of *C. fetus* subsp. *venerealis* to pregnant guinea pigs is a promising small animal model for the investigation of BGC abortion.

1. Introduction

Bovine genital campylobacteriosis (BGC) caused by *C. fetus* subsp. *venerealis* is a reproductive disease affecting many cattle herds (World Organisation for Animal Health, 2012). It has been associated with production losses in the Australian meat and dairy industry of up to 60% due to abortion and infertility in infected heifers (Clark et al., 1975). The bacterium is often asymptomatically carried by bulls, therefore diagnosis, treatment and control measures may be limited. It is venereally transmitted to heifers during mating. Heifers may either remain asymptomatic or experience late term abortions (Hum et al., 2009). This can lead to a continuous cycle of infection and infertility which may affect herd pregnancy rates (Clark, 1971).

Preventative and control measures for BGC exist in the form of controlled mating, antibiotic administration and vaccines. South American studies have found that vaccines are not always efficacious, with variation in protection offered by different vaccines against different strains (Cobo et al., 2003). This may be due to variation in strain virulence which is currently largely unknown. To define the level of pathogenicity and virulence variation present in *C. fetus* subsp. *venerealis* strains and to determine strain pathogenicity, a model for analysing and defining infection parameters is necessary. While in vitro models are much more ethical and financially viable, a greater understanding of the bacterial genes associated with virulence is needed and only single gene mutants can be examined in a single experiment (Kienesberger et al., 2007). In addition, in vitro models can only address one aspect of virulence i.e. epithelial cell invasion.

Guinea pig models have previously been used to assess variation in pathogenicity of *Campylobacter* species and strains including those of veterinary significance (Burrough, 2011; Burrough et al., 2009; Coid et al., 1987; SultanDosa et al., 1983). However, a model for determining *C. fetus* subsp. *venerealis* virulence has not been evaluated since its initial development 60 years ago (Ristic and Morse, 1953), while recent scientific advances (such as PCR) could improve model assessment. The model has not been used for the study of Australian strains, which may have different pathogenic characteristics to those in North America where the original study was undertaken (Ristic and Morse, 1953). An Australian based polymerase chain reaction assay could not differentiate UK *C. fetus* subspecies correctly hypothesizing that a unique clone was present in the UK (Hum et al., 1997; Willoughby et al., 2005). This may be due to different bacterial environments as well as antibiotic treatments which can impact bacterial genetic variation. The significance of a guinea pig infection model was shown in further studies using a variety of human *Campylobacter* species (but not *C. fetus*) with culture for diagnosis (Coid et al., 1987; Taylor and Bryner, 1984).

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Recent studies into *C. jejuni* infection models have included the examination of histological findings to substantiate the bacterial involvement in abortion (Burrough et al., 2009, 2011).

This study aimed to develop an animal model to assess variation in strain pathogenicity and subsequent abortions caused by different *C. fetus* subsp. *venerealis* strains. The model was established with accurate, defined parameters for the identification of dose, route and strains of *C. fetus* subsp. *venerealis* for further studies. The model was evaluated at a range of low, medium and high doses and using one of two different infection routes (intra-peritoneal and intra-vaginal). Subsequently culture, PCR and histology were compared to determine the presence of bacteria and the pathological changes associated with infection.

2. Materials and methods

2.1. Ethical statement

This study was approved by the University of Queensland Animal Ethics Committee (SVS070/10).

2.2. Study design

The study was designed as a controlled experimental trial with 35 female guinea pigs (*Cavia porcellus*) obtained from the University of Queensland's guinea pig breeding facility (Gatton campus) allocated to seven groups of five dams. Guinea pigs were assigned to groups in the order they became pregnant. Guinea pigs were housed in floor pens with wood shavings and had access to vitamin C supplemented water (1 g/l) and Barastoc guinea pig pellets (Ridley AgriProducts, Melbourne, Australia) *ad libitum* as well as fresh vegetables daily.

At the commencement of the study, 20 of the guinea pigs were tested for the presence of *C. fetus* subsp. *venerealis* or *Campylobacter*-like organisms (curved rod organisms growing under microaerobic conditions), by swabbing the vaginal area with a sterile cotton swab and placing it in PBS for transport to the lab where it was streaked out onto a sheep-blood agar (SBA) plate (Oxoid Australia, ThermoFisher Scientific, Waltham, MA, USA) and incubated in a microaerobic workstation (Don Whitely Scientific, Shipley, UK) at 37 °C for 72 h.

Abortion (defined as the expulsion of one or more foetuses or placentas) was the primary outcome. Guinea pigs were randomly housed in groups of 6–8 by blindly allocating dams to each pen with one male for mating purposes. They were then assigned to groups as they became pregnant based on closest gestational ages. Pregnant guinea pigs were inoculated via intra-peritoneal (IP) (groups 1–6) or intra-vaginal (IVA) (group 7) route between 5 and 6 weeks of gestation based on retrospective calculations of oestrus detection data. Each group consisted of five animals with an allocation ratio of 4:1 (test: control) per group (control receiving sterile broth).

2.3. Pregnancy determination

During the mating period, the guinea pigs were oestrus-detected by assessing the opening of the vaginal membrane (Stockard and Papanicolaou, 1919) once daily. When oestrus had not been detected for 21 consecutive days, trans-abdominal ultrasound using a MyLab 30 Vet ultrasound scanner with a 5–10 MHz linear probe (Esaote Pie Medical, Genoa, Italy) was performed. The presence of embryonic vesicles with foetuses and heartbeats confirmed pregnancy. The first five animals to fall pregnant were used in the first treatment group, with each subsequent set of five animals forming the following six treatment groups.

2.4. Inoculum preparation

Three *C. fetus* subsp. *venerealis* isolates were utilized in this study. Strain Q41 (ATCC19438), a control *C. fetus* subsp. *venerealis* was obtained from New South Wales Department of Primary Industries, and strains 258 and 540 were obtained from a bull prepuce abattoir survey with no traceability of bull infective status (Indjein, 2013). The three strains used were selected based on three features; (1) the molecular and biochemical profile matching *C. fetus* subsp. *venerealis* outlined by the World Organisation for Animal Health (World Organisation for Animal Health, 2012), (2) the viability of the bacteria 72 h after resuscitation based on bacterial growth and motility as seen microscopically, and (3) the survival of the bacteria when inoculated into 5 ml of guinea pig serum and incubated in a microaerobic workstation for 72 h.

The strains were resuscitated from storage at –80 °C by placing 200 µl of one of the stored media, either 85% FBP medium (Gorman and Adley, 2004) (0.025% ferrous sulphate w/v (Univar Australia Pty Ltd, Ingleburn, NSW, Australia), 0.025% sodium metabisulphite w/v (Sigma-Aldrich, St. Louis, MO, USA), 0.025% sodium pyruvate w/v (BDH ProLab, VWR International Pty Ltd, Murarrie, QLD, Australia) or 15% glycerol (AnalaR NORMAPUR, VWR International Pty Ltd), into 10 ml of vegetable peptone based Campylobacter broth consisting of 1% vegetable peptone no 1 w/v (Oxoid Australia, ThermoFisher Scientific), 0.2% sodium succinate w/v (Merck Australia, Merck KGaA, Darmstadt, Germany), 0.5% yeast extract w/v (Oxoid Australia, ThermoFisher Scientific), 0.5% sodium chloride w/v (Merck Australia, Merck KGaA), 0.0001% magnesium sulphate w/v (Univar Australia Pty Ltd), 0.5% calcium chloride w/v (Merck Australia, Merck KGaA), and 0.15% bacteriological agar w/v (Oxoid Australia, ThermoFisher Scientific). The broths were placed into a microaerobic workstation at 37 °C and assessed at 72 h for growth.

Viable strains were profiled using the Hum conventional PCR (Hum et al., 1997) and characterized biochemically according to OIE classification (World Organisation for Animal Health, 2012) – catalase, oxidase and growth in 1% glycine medium (Becton Dickinson and Company, Sparks Glencoe, MD, USA), growth at 42 °C, 25 °C and aerobically, susceptibility to 30 µg nalidixic acid and cephalothin (Oxoid Australia, ThermoFisher Scientific), H_2S production in a triple-sugar-iron (TSI) slope (Oxoid Australia, ThermoFisher Scientific) and in a 0.02% cysteine medium (Fluka analytical, Sigma-Aldrich) using lead acetate paper (Fluka analytical, Sigma-Aldrich).

The strains identified biochemically as *C. fetus* subsp. *venerealis*, were inoculated into 10 ml of fresh vegetable peptone based Campylobacter broth (as described above) and incubated microaerobically at 37 °C for 72 h. At 72 h, a serial dilution of 10^{-1} to 10^{-9} CFU/ml was prepared and plated in duplicate by placing 100 µl of the dilution onto an SBA plate and spreading it evenly across the surface. The plates were then incubated in the microaerobic workstation for 72 h. Subsequently, a comparative cell count using a Helber bacterial counting chamber (Hawksley, Sussex, UK) was carried out by creating a 1/10 dilution of the broth in 10% neutral buffered formalin (NBF) (10% w/v formaldehyde, Merck Australia, Merck KGaA) to fix the bacteria. A volume of 10 µl was placed into the central chamber of the counter, the cover slide added and viewed under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) at 400× magnification and the bacterial concentration calculated (Meynell and Meynell, 1970). Concentrations of the inoculum for groups 1–5 and 7 were based on growth at 72 h while group 6 was diluted with Campylobacter broth to achieve the desired concentration of bacterial colonies. Inoculum were streaked out just prior to inoculation onto two SBA plates, incubated at 37 °C either microaerobically or aerobically to ensure sterility of the broth.