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

Koya A.¹, ², de Wet S.C. ², Turner S.², Cawdell-Smith J.¹, Venus B.³, Greer R.M.¹, Lew-Tabor A.E.³, Boe-Hansen G.B.¹*

¹The University of Queensland, School of Veterinary Science, Gatton, Queensland, Australia
²Department of Agriculture, Fisheries & Forestry, Biosecurity Queensland, Coopers Plains, Queensland, Australia
³The University of Queensland, Centre for Animal Science, Queensland Alliance for Agriculture & Food Innovation, St Lucia, Queensland, Australia

* Corresponding author: Gry Boe-Hansen. Mailing address: The University of Queensland, Gatton 4343, Queensland, Australia. E-mail address: g.boehansen@uq.edu.au
Highlights

- Small animal model established for BGC evaluation of bacterial virulence
- Culture most consistent method for identification of BGC infection
- Histological evaluation showed placentitis and metritis as indicators of infection
- Strain virulence variation demonstrated, as assessed by culture and abortion rate

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 5 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.

**Keywords**

*C. fetus* subspecies *venerealis*, campylobacteriosis, placentitis, bovine, guinea pig
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 asymptptomatically 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, 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 hypothesising 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). Recent studies into *C. jejuni* infection models have included the examination of histological findings to substantiate the bacterial involvement in abortion (Burrough et al., 2011; Burrough et al., 2009).

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.
Materials and Methods

Ethical Statement

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

Study Design

The study was designed as a controlled experimental trial with thirty-five female guinea pigs (Cavia porcellus) obtained from the University of Queensland’s guinea pig breeding facility (Gatton campus) allocated to seven groups of 5 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, 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 5-6 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) and were inoculated as shown in Table 1.

**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.

**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), 0.025% sodium metabisulphite w/v (Sigma-Aldrich, MO, USA), 0.025% sodium pyruvate w/v (BDH
ProLab, VWR International Pty Ltd, Australia) or 15% glycerol (AnalaR NORMAPUR, VWR International Pty Ltd, Australia), into 10 ml of vegetable peptone based Campylobacter broth consisting of 1% vegetable peptone no 1 w/v (Oxoid Australia, ThermoFisher Scientific, MA, USA), 0.2% sodium succinate w/v (Merck Australia, Merck KGaA, Germany), 0.5% yeast extract w/v (Oxoid Australia, ThermoFisher Scientific, MA, USA), 0.5% sodium chloride w/v (Merck Australia, Merck KGaA, Germany), 0.0001% magnesium sulphate w/v (Univar Australia Pty Ltd, Australia), 0.5% calcium chloride w/v (Merck Australia, Merck KGaA, Germany), and 0.15% bacteriological agar w/v (Oxoid Australia, ThermoFisher Scientific, MA, USA). 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 characterised biochemically according to OIE classification (World Organisation for Animal Health, 2012) – catalase, oxidase and growth in 1% glycine medium (Becton, Dickinson and Company, MD, USA), growth at 42°C, 25°C and aerobically, susceptibility to 30 µg nalidixic acid and cephalothin (Oxoid Australia, ThermoFisher Scientific, MA, USA), H₂S production in a triple-sugar-iron (TSI) slope (Oxoid Australia, ThermoFisher Scientific, MA, USA) and in a 0.02%
cysteine medium (Fluka analytical, Sigma-Aldrich, MO, USA) using lead acetate paper (Fluka analytical, Sigma-Aldrich, MO, USA).

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, Germany) 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) at 400 x 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.

Experimental Procedures

Animal Inoculation

IP inoculation with 1.0 ml of the prepared strain was carried out using a 21G needle inserted slightly to the left of the midline of the abdomen after swabbing with iodine.

IVA inoculation was performed by placing the guinea pig on its back, anaesthetising with gaseous isoflurane using a Universal Vaporizer anaesthetic machine (Universal Vaporizer Support, Foster City, California, USA) and maintaining by mask with 1-2% isoflurane (Pharmachem, Kearny, New Jersey, USA) and 2-6 l oxygen per min. The vagina was gently opened using a sterile swab and a 3.0 mm tom-cat catheter (Henry Schein, New York, New York, USA) inserted into the cranial part of the vagina. The inoculum was then injected into the vagina, and the guinea pig maintained under anaesthesia for a further 5 min.

Animal Monitoring

Following inoculation, the animals were placed into individual pens and were checked twice daily (AM and PM) for any signs of systemic illness, such as increase in temperature or
respiration, vaginal opening, bleeding, or abortion. An
abdominal ultrasound was conducted every day (AM) to
determine number and health of foetuses, including
movement and foetal heartbeat.

Experimental Outcomes

The primary outcome was time to abortion or time to
euthanasia if no abortion had occurred by the end of the study
period. Animals displaying vaginal bleeding without foetal
death or expulsion of a foetus were closely monitored but
were not considered to be aborting. Upon the occurrence of
an abortion event (expulsion of one or more foetuses or
placentas), the dam was anaesthetized, sampled and
euthanized.

Secondary outcomes were bacterial culture; evidence of the
bacterium in tissues detected by PCR and histological evidence
of infection in the reproductive tract, including metritis or
placentitis.

Sampling Protocol

Immediately upon the detection of an abortion event or at the
end of the experiment timeline had no abortions occurred,
tissues were sampled for bacteria. Each guinea pig was
anaesthetized with gaseous isoflurane and maintained by
mask with 1-2% isoflurane and 2-6 l oxygen per minute. The abdominal area was swabbed with iodine and rear foot reflexes and breathing monitored before commencing with the sampling process. A longitudinal cut was made in the abdominal mid-line and the peritoneal cavity was swabbed. The left uterine horn was exteriorized and sampled using a swab through a small incision into the uterus. The uterine horn was then incised and the utero-placental junctions were swabbed. The foetuses in the horn were removed with the placenta and attachment site intact (incising through the uterine wall), and placed into individual petri dishes.

If the foetus had been expelled, the placenta with the attachment site was swabbed. The sampling protocol was then repeated for the right uterus horn. Due to the time sensitive nature of the procedure, plating could not be carried out at the time of sampling. To ensure the viability of the organism, all swabs were placed in an enrichment medium, Lander's (2.1% Mueller-Hinton broth w/v (Oxoid Australia, ThermoFisher Scientific, MA, USA), 0.5% bacteriological charcoal w/v (Amyl Media, Kings Langley, Australia), 7% lysed horse blood v/v (Oxoid Australia, ThermoFisher Scientific, MA, USA), 0.4% campylobacter growth supplement v/v (Oxoid Australia, ThermoFisher Scientific, MA, USA), 0.01%
cyclohexamide w/v, 0.002% vancomycin w/v, 0.001%
trimethoprim w/v and 2500 IU polymyxin B w/v (Sigma Aldrich, Missouri, USA)) until delivery to the laboratory (~85 km).

Cardiac puncture of the dam was performed and two drops of blood were placed directly into Lander’s transport medium for culture, while the remaining volume was placed into 9 ml culture tubes for extraction of serum for PCR. Immediately following cardiac puncture the guinea pig was euthanized with 2 ml of Lethabarb (Virbac, Carros, France). Amniotic fluid (AF) was collected and the foetus was incised to remove the foetal spleen and collect foetal stomach contents (FSC). AF and FSC samples were placed into individual Lander’s media. The placentas, foetuses and a 2 cm$^2$ piece of uterus tissue were placed into individual containers of 10% NBF for histology and a 0.5 cm$^2$ piece of the uterus horn and the spleens from the foetuses and dam were placed in individual microtubes on ice for PCR.

**Bacterial Culture**

Samples collected in Lander’s transport medium were incubated overnight at 37°C before streaking onto SBA plates and assessed at 72 h for growth of *Campylobacter*-like colonies. These colonies were then biochemically tested
according to international standards as published in the World Organisation for Animal Health manual (2012) to ensure the identity matched the biochemical profile of the inoculum.

**PCR Analysis**

The PCR previously described by Hum and colleagues (Hum et al., 1997) was carried out on DNA prepared from maternal and foetal spleen and dam sera from group 4-7, as well as a piece of the right uterus horn and each placenta from group 5-7. Tissue digestion and extraction was carried out using a Qiagen tissue kit (Qiagen, Hilden, Germany). Hum assay PCR primers were synthesised through Sigma (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) and PCR assays were undertaken in 20 µl reaction volumes using PCR reaction buffer with 25 mM Mg²⁺ (10× Hotmaster™ Taq buffer, 5 Prime, Quantum Scientific, Lane Cove West, NSW, Australia), 1U Hotmaster™ Taq DNA polymerase (5 Prime, Quantum Scientific), 1 µM of each forward and reverse primers, 1 µM dNTPs (Invitrogen) and 1.25 ng of *C. fetus* subsp. *venerealis* genomic DNA.
The reactions were amplified in a DNA Engine Thermal Cycler (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) using the following parameters: initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 2 min including a final extension of 10 min at the end. Products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA, pH 8) agarose gels containing 1% GelRed™ Nucleic Acid Gel Stain (Biotium, Jomar Diagnostics P/L, Stepney, SA, Australia) using either MassRuler™ Low range DNA ladder (Fermentas, ThermoFisher Scientific, MA, USA) or HyperLadder I (Bioline, Alexandria, NSW, Australia) and were visualised under Trans-UV light using the GelDoc™ XR+ system (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia).

**Histological Analysis**

All fixed tissue samples were trimmed through the centre-most point to allow maximum area for visualisation. Placentas attached to the uterus were trimmed to allow assessment of the utero-placental junction as well as the centre of the placenta (interlobium, labyrinth and subplacenta). Foetuses were sectioned three times, through the head, thorax and abdomen. While amniotic sac samples were collected when available, due to the low number this data is omitted. The
sections were then embedded in paraffin and routinely processed for haematoxylin and eosin (H&E) staining. Histologically, neutrophil aggregation and the presence of suppurative exudate, the first indicators of infection, were used to define the level of histological infection. Neutrophils were scored on a previously used semi-quantitative scale of 0 – 3 with score 0 = no neutrophils; 1 = mild neutrophil aggregation; 2 = moderate neutrophil aggregation; 3 = severe neutrophil aggregation as indicated by Figure 1 in the uterine tissue and Figure 2 in the placental tissue (Tursi et al., 2010).

**Statistical Analysis**

The time to abortion between groups was compared using Kaplan-Meier curves with the log rank test used to assess equality of survival distribution between the groups. Fisher’s exact test was used to compare the proportion of animals aborting, proportion of animals positive for *C. fetus* subsp. *venerealis* using culture or PCR and the proportion of animals with histological evidence of metritis or placentitis. Statistical analysis was performed using STATA (StataCorp LP, Texas, USA).

**Results**

**Baseline Data**
All animals were healthy, culture negative for *Campylobacter fetus* subsp. *venerealis* and were between 35-49 days pregnant when inoculated. Animals had a starting weight ranging from 862 g – 1020 g (mean = 973 g; standard deviation = 52 g). Vaginal swabs taken from 20 guinea pigs prior to inoculation were all negative on culture of *C. fetus* subsp. *venerealis*.

**Intra-peritoneal Challenge**

One hundred percent of the animals (excluding controls) in groups one and two (strain 258 $10^9$ CFU/ml) experienced abortion, compared to 0% in groups three (strain Q41 $8.4 \times 10^7$ CFU/ml), six (strain 540 $1.2 \times 10^4$ CFU/ml) and seven (strain 540 IVA $5.4 \times 10^7$ CFU/ml) ($p = 0.03$). In groups four and five (strain 540), 75% of animals experienced abortion. No control animals aborted (Table 1).

Time to abortion was not statistically different between groups inoculated with the same strains at slightly different concentrations as seen in groups one and two with strain 258 at $1.0$ and $1.5 \times 10^9$ CFU/ml ($p = 0.44$) and in groups four and five with strain 540 at $1.0 \times 10^8$ CFU/ml and $3.3 \times 10^7$ CFU/ml ($p = 0.62$) (Figure 4). Overall, the incidence of abortion differed between at least one of the groups 1-7 (group 1 vs 3, 6 and 7 p
= 0.0067, group 2 vs 3, 6 and 7 p = 0.0058, group 5 vs 3, 6 and
7 p = 0.0388, group 3 vs 4 p = 0.0401) (Figure 4).

Culture isolations from peritoneum, uterus, placentas and
amniotic fluid did not differ significantly between IP inoculated
groups 1-5 (p = 1.00) (Figure 3). However, groups 1-5 isolations
were significantly different to non-aborting groups six and
seven uterus (p = 0.03) and placenta culture compared to
groups one to five (respectively; p = 0.02, 0.01, <0.001, <0.001,
<0.001). Amniotic fluid isolations from group 7 was
significantly different compared to groups four and five (group
7 vs 1 to 6 respectively p = 0.14, 1.0, 0.15, 0.007, 0.0005 and
1.0). Isolations from foetal stomach fluid varied significantly
between groups two and four (p = 0.003), groups two and five
(p = 0.024) and between groups four and five and group 7
(group 4 vs 7 p = 0.002, group 5 vs 7, p = 0.02). Re-isolation of the
bacteria from all inoculated dams is shown in Figure 3 and
numbers of placental tissue, amniotic fluid and foetal stomach
content compared per group is displayed in Table 1.

Biochemical and molecular profiling for each strain matched
those of the inoculum. Comparative detection of the infectious
organism through culture, histology or PCR showed culture to
be the most consistent with re-isolation of the causative agent
in every aborting dam as well as the non-aborting dams in
group 3 (Figure 3). There was no difference in culture, histology or proportion of PCR positives at an animal level per group \((p = 1.00)\) (Figure 5).

Histological evidence of infection correlated with abortion events, with non-aborting groups (group 6 and 7) showing no neutrophil aggregation (Table 1). Strain 540 at concentrations of \(10^8\) CFU/ml showed a higher level (average score = 2.4) of neutrophil cell infiltration than at \(10^7\) CFU/ml (average score = 1). A high level of suppurative infiltration was seen in most abortive dam tissues, with haemorrhage and vacuolation visualized in only a few samples.

**Intra-vaginal Challenge**

None of the 4 intra-vaginally inoculated animals nor the control aborted within the 12 day (268 h) timeframe, and all animals were culture and histology negative while one sample (dam spleen) was PCR positive for *C. fetus* subsp. *venerealis* (Figure 5).

**Discussion**

This study found the pregnant guinea pig model to be a reliable and reproducible model for the study of *C. fetus* subsp. *venerealis* infection, strain virulence and dose dependency differences. The results of the study showed the
reproducibility of the model with concentrations above $10^7$

CFU/ml (strain 540 and strain 258). An increase in concentration of strain 540 by 1 log produced the same abortion rate (75%), with a decrease in the median abortion time by 20 h. Our results are consistent with a previous study where bovine strains of *C. jejuni*, *C. fetus venerealis* and *C. fetus* were tested at concentrations of $10^1$, $10^4$ and $10^8$ CFU/ml in pregnant guinea pigs (SultanDosa et al., 1983). Inoculated dams aborted at a rate of 60% (n=9) for *C. fetus* (1 strain), 13% (n=4) for *C. jejuni* (2 strains) and 73% (n=22) for *C. fetus* subsp. *venerealis* (2 strains) at all of the aforementioned doses within 21 days (SultanDosa et al., 1983). Abortions or abortive symptoms predominantly occurred within 2-17 days post-inoculation with an average of 7 days. The study also noted dose dependency, with the highest rate and shortest time to abortion produced at the highest dose amongst all strains used (SultanDosa et al., 1983).

In the present study, although none of the strain Q41 (ATCC19438) inoculated animals aborted, the bacterium was reisolated in all dams from a combination of 4 of the 7 culture sites (Figure 3; Table 1). This strain appeared to be less virulent than the previous strain (258) within the timeframe (7 days) resulting in an increase in the study endpoint to 12 days to
account for less virulent strains. Previous studies have used 2
days (Burrough et al., 2011), 12 days (Coid et al., 1987) and 21
days (Burrough et al., 2009) for *C. jejuni* and *C. coli*. However *C.
*fetus* subsp. *venerealis*, has been shown in cattle to require
longer incubation times as seen in aborting heifers which are
often affected in the third trimester of pregnancy (Hum,
2009). This was also seen in the initial *C. fetus* subsp.
*venerealis* guinea pig study with up to 34 days required as the
study endpoint (Ristic and Morse, 1953). The shortest effective
time-point was used in this study to avoid euthanizing fully
developed foetuses in non-aborting dams.

In cattle, inflammation in the uterus is associated with
infection as an indicator of campylobacteriosis associated
edometritis (Clark, 1971). Placentitis has been described as
an infection mechanism of *C. fetus* subsp. *fetus* in ewes
following bacteraemia, as well as in humans, as a mechanism
of abortion due to orally transmitted *C. jejuni* and *C. coli
(Skirrow, 1994). Burrough *et al* (2011a) using a *C. jejuni* guinea
pig model, showed that the organism had a high tropism for
the placenta, suggesting the presence of a ‘chemoattractive
placental factor’ which may account for the high level of
placental isolations seen in their study.
In cattle the bacteria are transmitted venereally, however, studies have shown that fertilization itself is not affected by \textit{C. fetus} subsp. \textit{venerealis} but rather fertility decreased by a reduction in implantation (Bielanski et al., 1994; Skirrow, 1994; Wilkie and Winter, 1971). These studies suggest that infection of the guinea pigs at the time of fertilization was not necessary to produce a comparative model for bovine infection. We found that inoculation by the IVA route was ineffective in producing infection or abortion. It is possible that organisms failed penetrate the cervix at the time of inoculation.

A more recent study into a virulence factor associated with \textit{C. jejuni} infection, \textit{luxS}, showed that it had the capability to affect motility, colonization and toxin production, and was directly linked to the bacteria’s ability to produce abortion in guinea pig and chicken models (Plummer, 2012). While the \textit{luxS} gene is present in \textit{C. fetus} subsp. \textit{venerealis} (Stynen et al., 2011) its involvement in bacterial pathogenicity is unknown. A disruption of \textit{virD4} (an essential component of the bacterial Type IV secretion apparatus) in \textit{C. fetus} subsp. \textit{venerealis} showed a 50% reduction in cell invasion \textit{in vitro} compared to the original wild type strain (Gorkiewicz et al., 2010). Such studies to date have been undertaken using \textit{in vitro} culture invasion assays but could also incorporate the use of gene
specific mutants to assist in the screening of potential virulence factors (Gorkiewicz et al., 2010; Kienesberger et al., 2007).

While the effect on fertilization could not be tested in this study as it was not possible to infect the guinea pigs at the time of mating, due to the similar end-point of this infection model and bovine pathogenesis – inflammation in the uterus and placenta causing early embryonic death and late abortions, this model is valid for use in studying bovine strain variations and infection mechanisms.

Culture has been, and still remains the gold standard for the diagnosis of BGC in infected cattle (World Organisation for Animal Health, 2012). Culture was reliable and consistent with high levels of re-isolation of bacteria from different sites in the animals. However, culture sensitivity at an animal level was lower than that of PCR in this study, with one sample in a dam inoculated via the IVA route positive by PCR but negative by culture or histology. This could be due to the infection becoming systemic and clearing through the spleen leaving residual DNA. However, due to the low number of samples tested by PCR in this study, further investigation and validation would be necessary before its recommended use in further studies.
This study has shown the difference between the ability of *C. fetus* subsp. *venerealis* strains to cause abortions at similar concentrations with strain Q41 (ATCC19438) having no effect while strains 258 and 540 had abortion rates of 100% and 75% respectively. Two studies using different strains (*C. fetus* and *C. jejuni*) at a concentration of $10^5$ CFU/ml found that IP injection of pregnant guinea pigs produced 83% abortions with 21 days with *C. jejuni* and 100% abortions within 12 days with *C. fetus* (Burrough et al., 2009; Coid et al., 1987). However, in the current experiment the *C. fetus* subsp. *venerealis* strain 540 at 1.2 x $10^4$ CFU/ml did not result in any abortions or show evidence of progression of infection. Genomic profiling may identify differences which may explain these results and also provide insights into genes which may be associated with higher virulence. The *C. fetus* subsp. *venerealis* strains used in this study appear to require a higher infective dose to produce abortions when compared to both *C. fetus* and *C. jejuni* (Burrough et al., 2009; Coid et al., 1987). The differences between previous studies and the present study include animal model species, infective dose, time to abortion, geographical origin of the strains and strain pathogenicity.

A limitation is that only three different strains were tested, strain Q41 (ATCC19438) only once, as well as the limited doses
tested. In contrast with other studies which used vaginal bleeding as the study endpoint, this study used abortion (defined as expulsion of one or more foetuses and/or placentas) as the endpoint (Burrough et al., 2011; Plummer, 2012). An important limitation on the interpretation of results occurred when samples were not readily collectable. In the instance of abortions, the dam would occasionally eat the placenta and/or part of the foetus and the amniotic sac become damaged and desiccated. With the occurrence of acute abortions, the presence of very small foetuses resulted in insignificant foetal stomach fluids and foetal spleen available for testing.

This study defined parameters for a route of infection, dose and study end-point to ensure the validity and reproducibility of a *C. fetus* subsp. *venerealis* infection model. It has also identified the most consistent sites for isolation of *C. fetus* subsp. *venerealis* and the most accurate method for evaluation of infection. The study has shown that the pregnant guinea pig model described here is promising for the assessment of further *C. fetus* subsp. *venerealis* strains. Defining the pathogenic and genomic variation of *C. fetus* subsp. *venerealis* strains may help ascertain the involvement of different bacterial virulence factors in BGC. This is a
valuable model for the testing of different strains to improve the understanding of bovine genital campylobacteriosis.

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LIST OF TABLES

Table 1. Summary of concentrations of three Campylobacter fetus subsp. venerealis strains used for inoculation of groups 1-7 of 4 guinea pigs and control group of 7 guinea pigs (one per group) each via either intra-peritoneal (IP) or intra-vaginal (IVA) route as well as time to study end-point, interquartile range of time to and percentage of abortions. Number of samples from IP inoculated dams (n=4 per group) which were culture positive for Campylobacter fetus subsp. venerealis in all foetuses (aborted and non-aborted) from groups 1-6. In the instance of abortions there was subsequent loss of samples due to fluid drying or dam eating all or part of placenta and foetus. Neutrophil aggregation scores for groups 1-7 as seen in uterus and placental tissues of inoculated dams. Foetal tissue samples (head, thorax, abdomen) showed no neutrophil infiltration suggestive of inflammatory response. Insufficient samples for an accurate representation of amniotic sac
infection were collected due to loss during abortion and/or desiccation and are therefore not shown.

LEDEND TO FIGURES

**Figure 1.** Uterine photomicrographs from *Campylobacter fetus* subsp. *venerealis* inoculated guinea pigs showing the inflammatory scale used for grading of neutrophil cellular infiltration. H&E stain; bar = 200µm. A – scale 0; normal endometrium (EM) and myometrium (MM). B – scale 1; scattered neutrophils within the EM. C – scale 2; neutrophils within the submucosa (SM) and EM and neutrophil exudate (NE). D – scale 3; widespread accumulation of NE along the EM and scattered within the MM. E - Non-infected control dam from group 1 showing EM tissue with no signs of inflammatory response (scale 0) X400. F – Infected dam from group 1 showing hyper-cellularity with evidence of inflammatory response, most notably infiltration of neutrophils and congestion of tissue (scale 3) X400.

**Figure 2.** Placental photomicrographs from *Campylobacter fetus* subsp. *venerealis* inoculated guinea pigs showing the inflammatory scale used for grading of neutrophil infiltration. H&E stain; bar = 200µm. A – scale 0; normal placental tissue
within the junctional zone (JZ), subplacenta (SP) and chorio-allantoic placenta (CAP).  

B – scale 1; scattered neutrophils within the JZ.  

C – scale 2; neutrophils within the SP and areas of neutrophil exudate (NE).  

D – scale 3; widespread accumulation of NE along and scattered within the SP.  

E - Non-infected control dam from group 4 showing normal yolk sac placenta with no signs of inflammatory response (scale 0)  

F - Infected dam from group 4 yolk sac placenta showing congestion of blood vessels, necrosis of epithelial cells and moderate inflammatory cell infiltrate comprising neutrophils and lymphocytes (scale 3) X400.

**Figure 3.** Percentage of samples from each of the three anatomical sites of inoculated dams which were culture positive for *Campylobacter fetus* subsp. venerealis in aborting and non-aborting instances (n=4) in groups 1-7 (see Table 1).  

No blood samples were collected in groups 1-3.

**Figure 4.** Kaplan-Meier survival estimates of groups 1-7 showing abortion incidences to end-point with log rank for equality of survivor function chi² = 24.16 and Pr>Chi² = 0.0005.

**Figure 5.** Comparison of bacterial presence detected through culture, histology and PCR in inoculated animals (n=4) of groups 1-7 (see Table 1). The criterion for a positive result was a minimum of one positive sample per animal for each test.
Culture results are from isolations in peritoneum, uterus, placenta, blood, amniotic fluid or foetal stomach contents. Histological findings were associated with metritis or placentitis per animal and positive PCR results for uterus, spleen, serum or placenta for each inoculated animal. PCR was not done on samples in groups 1-4.

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control group consisted of 1 animal per group 1-7 inoculated with sterile broth via the same route and endpoint.

* h – hours; IQR – interquartile range; N/A – not applicable; CFU/ml – colony forming units per millilitre; AF – amniotic fluid; FSC – foetal stomach contents; n – number

\(^a\) time to abortion significantly different to group 3, 6 and 7 (p=0.0067); \(^b\) – time to abortion significantly different to groups 3, 6 and 7 (p=0.0058); \(^c\) – time to abortion significantly different to groups 3, 6 and 7 (p=0.0388).