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Identification of *Chlamydia abortus* and the development of lesions in placental tissues of experimentally infected sheep

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Abstract

*Chlamydia (C.) abortus* is a major cause of infectious abortion in sheep in many countries. Twenty-one pregnant sheep were experimentally infected intranasally with *C. abortus* at 70 days of gestation (dg). Thereafter, a number of animals were killed at weekly intervals and a post mortem examination was carried out. Evidence of chlamydial infection in the placenta was determined by isolation of the bacterium by tissue culture and detection of *C. abortus* DNA by real-time polymerase chain reaction (real-time PCR). In addition, histopathological changes in the placenta were assessed, as was the detection of chlamydial antigen by immunohistochemistry (IHC). Evidence of placental infection was observed as early as two weeks after inoculation, and while only relatively low numbers of bacteria were isolated by culture and/or detected by real-time PCR prior to 113-114 dg, at 119-121 dg, it was more numerous. This study, using the four criteria for assessment of infection, showed that while *C. abortus* gained access to the placenta as early as 85 dg, characteristic histopathological changes were not apparent until 119/121 dg. While the chronology of when the bacterium arrived in the placenta and subsequent lesion development is remarkable for its consistency this paper provides more reliable
data on the former which in turn now allows study of the factors that permit its access to
this tissue and govern its multiplication and the ensuing triggering of damage.

**Keywords:** *Chlamydophila abortus; ovine placenta; cell culture; real-time PCR; pathology; IHC*

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1. Introduction

*Chlamydophila (C.) abortus*, the causative bacterium of ovine chlamydial abortion (syn. ovine enzootic abortion, enzootic abortion of ewes), was first identified by Stamp and colleagues (1950) and is recognised as a major source of abortion and lamb loss in many sheep-rearing countries (Longbottom and Coulter, 2003; Aitken and Longbottom, 2007). Initial infection with *C. abortus* usually goes unnoticed, although a transient febrile response and general malaise may occur soon after experimental infection (Buxton et al., 1990; Papp and Shewen, 1996a; Sammin et al., 2006).

*Chlamydophila abortus* often resides in a latent form before it is released to infect the gravid uterus, but the site of latency and the trigger to its release are not known. The first clinical evidence that animals are infected occurs during the current or subsequent pregnancy. Typically, ewes will abort up to 2-3 weeks before term, with considerable lamb mortality in some flocks, especially in the first 2-3 lambing seasons after exposure to infection (Longbottom and Coulter, 2003). Affected placental membranes, contaminated newborn lambs and the ewes’ vaginal discharges may all be a source of infection for naïve animals with the natural route of transmission considered to be by
inhalation or ingestion of the organism in these materials, or on contaminated pasture (McEwan et al., 1951; Wilsmore et al., 1984). This conclusion was supported by Jones and Anderson (1988) who inoculated six ewes over the tonsillar crypts at 70 days gestation (dg) with \textit{C. abortus}. Abortion occurred in four ewes and chlamydial antigen was detected in their foetal tissues, leading the authors to conclude that the tonsil may be a natural site of access for chlamydial infection.

The appearance of placental lesions was shown to occur after 90 dg, following subcutaneous inoculation of \textit{C. abortus} (Buxton et al., 1990). The aim of the present study was to examine in more detail the time of onset and subsequent development of placental lesions in ewes experimentally infected with \textit{C. abortus} at 70 dg.

2. Materials and Methods

2.1. Preparation of inocula

\textit{C. abortus} strain S26/3 was grown in embryonated hen's eggs and infected yolk sacs prepared as previously described (McClenaghan et al., 1984). Egg-grown material was diluted with sucrose-phosphate-glutamate (SPG) (Spencer and Johnson, 1983) medium to provide a challenge inoculum containing $2 \times 10^6$ inclusion forming units (ifu)/ml of \textit{C. abortus}. A similar control inoculum was prepared from uninfected yolk sacs.

2.2. Animals, experimental design and tissue sampling

Twenty-one pregnant conventionally reared Scottish Blackface sheep aged 3-5 years old (group 1), seronegative to \textit{C. abortus} and \textit{Toxoplasma gondii}, were each
inoculated, subcutaneously (sc) over the left prefemoral lymph node, with 1 ml of 2 x 10^6 ifu of C. abortus, isolate S26/3 at 70 days gestation (dg) (Table 1). The experiment was carried out in accordance with the requirements of the Moredun Research Institute’s ethics committee and with the agreement of the UK Home Office Inspectorate. Ultrasound scanning was carried out prior to this, to determine the number of foetuses in each sheep. In group 1, three ewes were killed (with intravenous barbiturate) two weeks later and groups of three were similarly killed at weekly intervals thereafter; with two each carrying twins and one carrying a single lamb. Seven control ewes, carrying twins (group 2), were each inoculated sc with 1 ml of control inoculum at the same site and at the same stage of pregnancy as group 1 ewes, and one animal was killed at each of the time-points described above.

At the post mortem examination, 10 placentomes (P1-P10) with associated foetal membranes were collected from each placenta. Pooled samples (P1-P5 and P6-P10) from each were collected aseptically and placed in chlamydial transport medium for culture and also into a sterile container for detection of bacterial DNA by a real-time polymerase chain reaction (PCR) (see below). Larger samples, individually labelled (P1 to P10) were also placed in 10% formal saline for histopathology.

2.3. Detection of C. abortus

2.3.1 Culture

Placental samples were weighed and aseptically ground up with SPG medium, supplemented with 10% foetal calf serum and antibiotics such as streptomycin and gentamycin. Ten-fold serial dilutions were prepared in complete RPMI medium.
containing 2% (v/v) foetal bovine serum (FBS) and 1 µg/ml cycloheximide (Sigma-Aldrich Company Ltd., Poole, United Kingdom). Diluted material was inoculated onto confluent McCoy cell monolayers grown in complete RPMI medium containing 5% (v/v) FBS on coverslips in trac bottles (Barloworld Scientific, Stone, United Kingdom). Following centrifugation (3,000 × g at room temperature for 2 × 15 min), tracs were incubated at 37°C under 5% CO₂ humidified atmosphere for 72 hrs. Coverslips were then fixed in methanol, stained by Giemsa Gurr (Merck Ltd., Poole, United Kingdom), and examined for the presence of chlamydial inclusions by light microscopy. Results were expressed as the number of *C. abortus* ifu per gram of tissue.

2.3.2 Real-time PCR

Real-time PCR amplification of *C. abortus* DNA, using *C. abortus*-specific major outer membrane protein primers, was carried out on placental samples as described by Livingstone et al (2008). The real-time PCR was only performed on samples where substantial numbers of the organism could not be detected by culture. Briefly, 200 µl of homogenised placental samples in SPG medium were centrifuged and pellets extracted using a DNeasy Tissue Kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. PCR reactions were then performed in a 96-well plate (AXYGEN Scientific, Union City, USA) with reaction mixtures consisting of 12.5 µl of 2 x TaqMan universal PCR master mix (Applied Biosystems, Warrington, UK), 300nM concentrations of each primer, 250 nM concentration of fluorescent probe and 1 µl of extracted DNA, made up to a final volume of 25 µl with water. Amplification and detection were performed with an AB Prism 7000 sequence detection system (Applied Biosystems). The number of
genomes was calculated for each sample using Avogadro's number by including a *C. abortus* genomic DNA standard curve and the results expressed as the number of genomes per 200 µl of homogenized sample.

2.3.3 Histopathology

Placentomes were fixed in 10% formal saline for 4-10 days and then trimmed, processed through graded alcohols and a xylene step and embedded in paraffin wax. Serial sections 5 µm thick were cut from each placentome, and one from each sample was stained with haematoxylin and eosin (HE) and examined for the presence of pathological changes.

2.3.4 Detection of *C. abortus* in tissue sections by immunohistochemistry (IHC)

Serial sections of placentomes were treated using an immunohistochemical method for the detection of *C. abortus* (Buxton et al., 1996). After dewaxing, sections of placentomes were incubated with a mouse monoclonal antibody (mab) against the LPS of strain S26/3 of *C. abortus* (mab 13/5; Buxton et al., 1996) and then treated using the avidin-biotin complex (ABC) technique (Vectastain Elite ABC kit; Vector Laboratories, Peterborough, United Kingdom). The reaction was developed with 0.05% diaminobenzidine tetrahydrochloride, 0.01% hydrogen peroxide in 0.1M Tris buffer, pH 7.2 for 2-5 min. Sections were then washed in water, counterstained with haematoxylin, dehydrated in graded alcohols, cleared using xylene and mounted under coverslips, using a xylene-based mounting medium.
3. Results

3.1. Detection of C. abortus

3.1.1 Culture

In group 1, C. abortus was recovered from placental tissue from ewes at 85 dg (ewe 1) and 92 dg (ewe 4) (Table 1). C. abortus could not be cultured from tissues at 99-100 dg but it was recovered at a low level at 106 dg (ewe 10). Large numbers of organism were recovered from animals at 114 dg (ewes 14 and 15), but the greatest number of C. abortus organism, detected by culture, was in samples at 119-121 dg (ewes 16, 17 and 18). Organism could only be isolated from one of the three animals at 127-128 dg (ewe 21). C. abortus was not isolated from any placental samples from group 2 (Table 1).

3.1.2 Real-time PCR

Chlamydophila abortus DNA was detected in all of the placental samples selected for testing from group 1 that were positive by culture (Table 1). In addition, C. abortus DNA was detected in three samples, in which the organism was not detected by culture, at 106 dg (foetus 1, P6-P10), 107 dg (foetus 2, P1-P5) and 128 dg (foetus 1, P1-P5). C. abortus DNA was not detected in any placental samples from group 2 (Table 1).

3.1.3 Histopathology

In group 1, a small area containing a light scatter of polymorphonuclear neutrophils along with a mixture of mononuclear cells, that included macrophages, was present in the foetal placental connective tissue of one foetus at 106 dg, 36 dpi (ewe 10)
(Figure 1A). Similar traces of inflammation were seen at 113-114 dg, 43 dpi, in a small proportion of placental foetal villi from two of the three ewes (ewes 14 and 15) (Table 1). More obvious lesions characteristic of *C. abortus* infection (Buxton et al., 1990; Sammin et al., 2006) were seen in the majority of the placental samples examined at 120 dg (Figure 1B) and in one animal at 128 dg (ewe 21). These included necrosis and loss of the foetal chorionic epithelial cells, vasculitis and thrombosis of foetal blood vessels in the hilar region of the placentome and associated marked infiltration of the foetal placental connective tissue by mononuclear inflammatory cells and polymorphonuclear neutrophils. No histopathological evidence of infection by any other abortifacient pathogens was detected in either group, and all placental samples from group 2 were normal.

3.1.4 Detection of *C. abortus* in tissue sections by IHC

*Chlamydia abortus* was detected by IHC in the placentae of two animals from group 1 at 85-86 dg (ewes 1 and 2) (Table 1), in the form of intracytoplasmic inclusions in chorionic epithelial cells (Figure 1C). No positive labelling was detected at 92-93 and 99-100 dg (Table 1). Chlamydial antigen was observed in one animal at 106 dg (ewe 10). *Chlamydia abortus* was detected in the chorionic epithelial cells in the placentae of two animals at 114 dg (ewes 14 and 15) and in all three ewes at 119-121 dg (Figure 1D). In animals with more advanced lesions, diffuse and granular labelling was associated with destruction of the chorionic epithelium and adjacent maternal caruncular septal tissue. At 128 dg positive labelling was detected in one ewe (ewe 21), with trace amounts
observed in a second (ewe 20). *C. abortus* was not detected in any of the placental samples from animals in group 2.

4. Discussion

The placenta is the primary target for *C. abortus* in ovine chlamydial abortion (Buxton et al., 1990; Papp and Shewen, 1996b; Sammin et al., 2006). In the present study, the sc inoculation of *C. abortus* at 70 dg caused placental infections that could be detected by tissue culture, real-time PCR, the presence of histopathological changes characteristic of ovine chlamydial abortion and by labelling of *C. abortus* antigen by IHC. In most instances, the successful recovery of *C. abortus* in tissue culture was mirrored by the detection of *C. abortus* DNA in associated placental samples (Table 1) with the organism detected as early as 15 days after inoculation in one of three ewes. Subsequently, infection was detected at 92-93 dg, 106-107 dg, 113-114 dg and 119-121 dg. At 128 dg it was only detected in one of three ewes. In this study, the use of the real-time PCR has shown it to be a valuable tool for the detection of *C. abortus* being at least as sensitive as tissue culture. Although with detection in instances that were negative by culture it is not possible to conclude whether it is detecting DNA of the non-living organism.

The combination of detection of *C. abortus* in ewes by IHC, culture and real-time PCR provides a reliable profile of when infection gains access to the placenta, to compare with, when inflammatory lesions start to develop. The latter changes, characteristic of *C. abortus* infection (Buxton et al., 1990; Sammin et al., 2006), were well established at
119-121 dg although the organism was detected from 85 dg to 107 dg in four of the 12 ewes sampled, with only a trace of inflammation detected 36 dpi at 106 dg. In an earlier study (Buxton et al., 1990), the organism was isolated at 95 dg from one ewe inoculated at 60 dg and at 103 and 105 dg after inoculation at 90 dg but inflammatory lesions were not detected until 25 dpi at 115 dg. Infection therefore appears to gain access to the placental chorionic epithelium within 15 days of inoculation but in both studies its multiplication was at a relatively low level until after 115 dg. As the foetus and its placenta become progressively more immunocompetent after 80 dg (Nettleton and Entrican, 1995), investigation of the factors governing chlamydial multiplication and the initiation of inflammatory changes could be very illuminating.

Whether infection is acquired before or during pregnancy, there are no clinical signs until the days before abortion occurs (Buxton et al., 1990; Papp et al., 1993). In the present study at 127-128 dg, in one animal, no evidence of exposure to *C. abortus* was detected and in another only a trace of immunohistochemical labelling of *C. abortus* was demonstrated (Table 1). The reasons for this are unclear, but the fact that not all experimentally infected sheep in this study showed evidence of infection would seem to mimic the situation in the field, where not all animals exposed to infection go on to abort (Longbottom and Coulter 2003).

In pregnant animals, recrudescence of *C. abortus* may be linked to physiological changes that occur naturally in the placenta where, at around 60 dg, maternal haematomata appear in the placentomal hilus as a result of normal invasion of the caruncular stroma by foetal chorionic villi (Sammin et al., 2008). This could allow any *C.
abortus circulating in the ewe's blood direct contact with the chorionic epithelium thus
initiating infection (Buxton et al., 1990).

Thus experimental infection of pregnant sheep, at mid-gestation, with C. abortus was successful and infection of the placenta and development of lesions were confirmed as being characteristic of ovine chlamydial infection. While infection was detected as soon as 85dg (15 dpi) in the placentome it was not until around 120 dg that characteristic lesions were observed. Now that the timing of entry of Chlamydophila to the placenta is established, the underlying factors that influence its arrival and its multiplication and the consequent development of placentitis can be studied.

Conflict of interest statement

None of the authors (S.W. Maley, M. Livingstone, S.M. Rodger, D. Longbottom and D. Buxton) has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the paper entitled “Identification of Chlamydophila abortus and the development of lesions in placental tissues of experimentally infected sheep”.

Acknowledgements

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References


Papp, J.R., Shewen, P.E., 1996a. Pregnancy failure following vaginal infection of sheep with *Chlamydia psittaci* prior to breeding. Infect. and Immun. 64, 1116-1125.


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<th>Foetus 1 Real-time PCR $^{c}$</th>
<th>Foetus 1 Pathology $^{d}$</th>
<th>Foetus 1 IHC $^{e}$</th>
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- a = 2 x 10$^6$ ifu of C. abortus subcutaneously at 70 dg, b = Uninfected yolk sac
- c, results expressed as the number of C. abortus ifu per gram of tissue, d = placentomes 1-5 (pooled sample), e = placentomes 6-10 (pooled sample), f, results expressed as the number of genomes per 200 µl of homogenized sample
- g, - negative, +/- trace pathological changes, + mild pathological changes and ++ extensive pathological changes
- h, - negative, +/- trace amounts of antigen, + positive for antigen in >1 area and ++ positive for antigen throughout and associated with extensive placental pathology
Table 1. Detection of *C. abortus* in placental tissue samples by recovery in culture, real-time PCR, occurrence of pathological changes and labelling of antigen by immunohistochemistry.
Figures

Figure 1. Pathological changes and chlamydial antigen in *C. abortus*-infected ovine placenta. A. Ewe at 106 dg (36 dpi) with mild inflammation in the foetal connective tissue and including polymorphonuclear neutrophils (arrows), HE. x 100. B. Ewe at 120 dg (50 dpi), showing loss of chorionic epithelial cells with intense inflammation (i) just beneath the basement membrane. Note also the desquamated necrotic debris (d) on the right of the picture and vasculitis (v) on the left, HE. x 100. C. Immunohistochemical labelling of *C. abortus* LPS in sheep placenta of ewe at 85 dg (15 dpi). Cross section of placentome showing positively-labelled chlamydial inclusions (brown) in the chorionic epithelial cells (arrows). IHC. x 200. D. Cross-section of the ovine placentome of ewe at 119 dg (49 dpi), showing large areas labelled positive (brown) for *C. abortus* antigen. Note the area of necrosis (n), IHC. x 100.
Figure 1c
Figure 1d