Studies on Carrier State of *Chlamydophila abortus* in Naturally Infected Sheep

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Abstract

The purpose of the study was to establish whether or not *C. abortus* is shed through the reproductive tract following infection under natural conditions. Two farms where enzootic abortion was endemic were investigated by sequential collection of vaginal secretions, faeces and serum samples from ewes which had chlamydial abortion six months earlier. PCR was used as a confirmatory tool after extracting DNA from faeces and vaginal secretions using primers specific to the MOMP gene. None of the samples tested were positive, suggesting that either there were no EBs of *C. abortus* in the samples or there were non-infectious forms (RBs) which could not be detected by DNA amplification. Real-time PCR analysis of placental samples identified very few or no chlamydial genomes, which contrasted significantly with samples taken at the time of abortion. The *C. abortus* was detected by PCR for the first time in milk samples collected from ewes with EAE history using the IMS technique. The serum samples from these ewes showed significantly higher chlamydia-specific IgG titre during the period of peri-ovulation. In terms of flock management, the products of abortion should be considered the major and principal source of infection for transmission to naïve ewes.

Introduction

Ovine Enzootic Abortion (OEA) is one of the most common contagious disease affecting sheep and goats due to *Chlamydophila abortus* (*C. abortus*). Unlike other bacteria, the chlamydial development life cycle is characterized by two distinct developmental forms, elementary bodies (EBs) and reticulate bodies (RBs) respectively. The Elementary body is infectious, with rigid cell wall, which contains the major outer membrane protein (MOMP) and other proteins. The reticulate bodies are non-infectious, metabolically active forms of chlamydiae capable of synthesizing DNA, RNA and proteins. Enzootic abortion in sheep (EAE) causes a major financial loss worldwide and represents a significant zoonotic risk. It is clinically characterized by abortion and weak neonates in small ruminants. Pregnant sheep initially infected with *C. abortus* either abort late in gestation or give birth to weak or stillborn lambs as a result of placental pathology associated with infection (14). Using specific real-time PCR assay, few chlamydial genomes could be detected in vaginal swabs taken from post-abortion ewes at oestrus. Few genomes could also be detected from vaginal, cervical tissue samples and lymph nodes taken post-mortem (8).

Because parturition or abortion in *C. abortus* infected ewes is usually accompanied
by the passage of an infected placenta and or foetal tissues, the main concern of transmission of the infection to susceptible ewes in the periparturient period when the lambing environment may become contaminated with diseased tissues (18). Chlamydia-induced abortion in sheep was recognized to stimulate an immune response that protected against subsequent abortion (9). Ewes that experienced abortion as a result of experimental infection with \textit{C. abortus} maintained an elevated systemic antibody response to the organism and this was associated with a chronic reproductive tract infection (13) Since future fertility was apparently not compromised, ewes that aborted are usually retained and reintroduced to the flock. Identification of those carrier ewes could aid the development of a management strategy to eliminate continued disease outbreaks in flocks. The purpose of the present study was to establish whether or not \textit{C. abortus} is shed through the reproductive tract following infection under natural conditions.

\textbf{Material and Methods}

\textbf{Experimental design}: Two sheep farms where enzootic abortion was endemic were selected to study the carrier state of \textit{Chlamyphila abortus}. Fourteen ewes confirmed to have had aborted due to the natural infection of \textit{C. abortus} six months earlier were randomly selected for the study and was investigated by sequential collection of vaginal secretions, faeces and serum samples.

Synchronization of oestrus cycle was carried out in all the fourteen ewes by inserting vaginal progesterone impregnated sponges and by parenteral administration of pregnant mare serum gonadotrophin (PMSG). Blood and faecal samples were collected on the day of sponge insertion. Thirteen days later the sponge was removed before injecting each ewe with PMSG. Immediately Coagulated blood, faeces and vaginal samples were collected. Again 2 and 4 days later vaginal swabs were collected. The ewes were euthanized 33 days later and the fallopian tube, mid uterus, and vagina were used for chlamydial isolation. All samples were stored at –80 °C until further use.

\textbf{Collection of samples}

\textbf{Faecal samples}: Fresh rectal faeces collected (0.1 to 25 g) were placed in transport medium mixed with a rotomixer and centrifuged at 1000 xg for 10 min. Twenty µl of the supernatant were then used to inoculate a monolayer of McCoy together with culture medium containing 200 µg/ml of gentamycin (15).

\textbf{Reproductive tract samples}: The sections of the vagina distal, the mid region of the uterus and the oviduct were obtained under aseptic conditions and immersed into transport medium. The samples were cut up into smaller pieces with scissors and broken further with mortar and pestle. After centrifugation at 1000 g for 10 min, 100 µl of the supernatant were used to inoculate McCoy cells (12)

\textbf{Vaginal swabs}: Vaginal swabs for chlamydial isolation and PCR were collected after gently inserting sterile swabs into the vagina and rotating them in both directions. Immediately after sampling, the tip of the vaginal swab was immersed in transport medium, mixed with rotomixer and centrifuged at 1000 x g for 10 min. The supernatants were then used to inoculate McCoy cell monolayers and for DNA extraction. The samples were kept at –80 °C until used.

\textbf{Bacterial identification and isolation}

McCoy cells, grown in 24-well plate monolayer were used for the isolation. Inoculated monolayers were examined for the presence of inclusion bodies after staining cover slips with Diff Quick stain at 48-72 hours post inoculation. Some
monolayers were frozen at -20 °C, then thawed at 37°C, and the inclusions were disrupted by shaking with glass beads for 1 min before centrifugation at 5000 x g for 10 min at 4°C. The supernatant was collected and the pellets were resuspended in sucrose phosphate glucose (SPG). The suspension was centrifuged again after brief sonication. The supernatant was pooled and centrifuged at 30,000 g for 30 min at 4°C (Sorvall instrument, DuPont, USA). The pellet obtained after ultracentrifugation was used to inoculate fresh McCoy cell monolayers. After three inoculation cycles in fresh cells, the media and cell pellet were tested for chlamydia-specific lipopolysaccharide (LPS) by using the Clearview Chlamydia test kit (Unipath, Inc., Nepean, Ontario, Canada). Direct faecal samples smears were examined for the presence of MZN-positive elementary by Modified Zeihl Nelson (MZN) stain.

**Immunological Technique**

*Enzyme linked immunosorbent assay (ELISA)*: The ELISA test was performed on serum samples collected before insertion of progesterone sponge insertion and 13 days later (sponge removal and PMSG injection).

**Polymers Chain Reaction (PCR)**

*DNA extraction of vaginal and faecal swabs*: The swabs collected in Chlamydia transport medium (CTM) were centrifuged at 2000 rpm for 10 minutes. About 400 µl of the supernatant was then pelleted by centrifugation for 10 minute at 1200xg. After resuspending the pellet in 180 µl of ATL solution (supplied in the QIAamp DNA mini kit) and adding 20 µl of proteinase K, the samples were incubated at 65°C overnight. The micro-centrifuge tube was centrifuged before adding 200 µl buffer AL to the sample, then vortex mixed for 15 sec and incubated at 70°C for 10 min. The tube was centrifuged and 200 µl of ethanol (96-100%) was added and mixed. The mixture was then carefully poured in the QIAamp spin column. The cap was then closed and centrifuged at 6000 x g for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube and the other tube containing the filtrate discarded. 500 µl of buffer AW1 was added and centrifuged at 6000 x g for 1 min and the tube containing the filtrate was discarded. Then, 500 µl of Buffer AW2 was added and centrifuged at 20,000 x g for 3 minute and repeated as above. 200 µl of Buffer AE or distilled water was added. After incubation at room temperature for 1-5 min, the mixture was centrifuged at 6000 x g for 1 min and the DNA extract was stored at -20°C until used.

**DNA amplification**: DNA was amplified as described by Creelan and McCullough (2000), using Clone 8 forward primer, 5’ TGG TAT TCT TGC CGA TGA C 3’; and clone 8 reverse primer, 5’ GAT CGT AAC TGC TTA ATA AAC CG 3’ for the *omp* gene of *Chlamydophila abortus*. 3 µl of the DNA sample was added to 47 µl of a reaction mixture containing 1xPCR super mix (Life Technologies (20 mM Tris-HCl pH 8.4, 5 mM KCl, 1.5mM MgCl2), 200 µM deoxynucleoside triphosphates, 2 Units Taq DNA polymerase and 1 mM of each primer. The reaction mixture was overlaid with 30 µl of mineral oil and initial denaturation at 95°C for 5 minutes followed by 40 cycles of; 1 minute of at 94°C, 1 min of at 45°C, 2 minutes at 72°C and final 7 min extension at 72°C. The amplified product was analysed by subjecting 5 of PCR product to electrophoresis for 45 minute at 100 V on a 1 percent agarose gel. The gel was then stained with ethidium bromide and illuminated by ultraviolet light. Molecular weight markers X 174(Sigma) were included in every gel.

Studies on carrier state of *Chlamydomphila abortus*
Statistical analysis

The antibody titres to *C. abortus* at two occasions of oestrus synchronization (day 0 and 13-days later) were compared by paired t test.

Results

**Detection of chlamydiae:** At various occasions of oestrus synchronization (Day 0 and 13-days later) *C. abortus* was not isolated or specific antigen was detected in the vaginal and faecal samples collected on the day of progesterone sponge insertion (Day 0) or on the day of sponge removal (day 13).

**Detection of chlamydial LPS:** Chlamydia–specific LPS were not detected by the Clearview test in the samples collected before insertion or in those obtained after sponge removal and PMSG injection. Samples continued to be negative after the third passage on McCoy cell tissue culture. PCR results using MOMP specific primers were also negative.

**Systematic antibody response:** Chlamydia-specific IgG antibodies were detected in the sera tested by ELISA. The mean IgG antibody titre of the serum samples obtained from the ewes on the day of sponge insertion was 2.05 (log_{10}), and those obtained after the removal of the sponge was 2.93 log_{10} (Table 1). The rise in antibody titre following treatment with progesterone was statistically significant (p<0.001)

Discussion

In the present study the possibility of a carrier state following chlamydial abortion in two farms where enzootic abortion was endemic has been investigated by sequential collection of vaginal secretions, faeces and serum samples from ewes which had chlamydial abortion six months earlier. *C. abortus* was not isolated after three passages in any of the samples tested and chlamydial antigens were not detected by Clearview in the faecal samples and vaginal mucus obtained before or after insertion of the progesterone sponges. However, significant levels of antibodies were detected by ELISA in serum samples obtained before and after insertion of the sponges, with titres significantly rising after insertion.

In earlier studies, clinical, serological and immunological tests revealed that the lowest number of infected sheep was registered in animals younger than 18 and highest in animals aged 18 to 24 months. In sheep aged more than 3 years, titre of antibodies was significantly reduced. Furthermore, in sheep which aborted, the infection level was 2.5-fold higher as compared to sheep which didn’t abort. The highest prevalence of chlamydia (66.7%) was registered in placentas of sheep which aborted (1). These results contradict those reported by Papp et al. (13), who detected LPS but not live chlamydiae, after the third passage of vaginal swabs obtained from chronically infected ewes. The specific serum antibodies to Chlamydophila abortus was present

<table>
<thead>
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<th>Animal No</th>
<th>Sponge insert-ion</th>
<th>Sponge removal</th>
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<tbody>
<tr>
<td>90</td>
<td>0.9</td>
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</tr>
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<td>P (&lt;0.05)</td>
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in 2,360 out of 20,878 sheep sera examined (11.7%), and in 85 out of 1,162 examined goats (7.7%) in slovek republic (2).

The lack of detection of chlamydial antigen by MZN or by culture is consistent with that reported by Clarkson and Philips, (3), who could not detect C. abortus in the faecal samples obtained from ewes in farms where enzootic abortion was endemic. However, other workers have reported that C. abortus is occasionally isolated from faeces (16). It appears that C. abortus is not excreted in the faeces of naturally aborting ewes but further studies are needed to examine flocks where enzootic abortion is present and for the possible role of faeces as source of infection, as some enteric isolates were reported to be capable of causing abortion in pregnant ewes after intravenous injection (15).

In the present study attempts to demonstrate chlamydial antigens by immunohistochemical staining of sections of the reproductive tract obtained from ewes six months after chlamydial-abortion were also unsuccessful. This is also in contradiction to the observations of Papp et al (11), who reported the demonstration of chlamydial antigens in vaginal and cervical samples obtained from chronically infected ewes using immunohistochemistry. However, their samples were obtained from ewes that had experienced C. abortus induced abortion 3 years earlier. Whereas in the present study the samples were obtained during the second oestrus cycle, six months after abortion due to C. abortus.

The Clearview Chlamydia test kit is reported to provide fast and easy-to-use method for the detection of chlamydiae. It has high sensitivity, which makes it a useful screening tool, but it is not species-specific as the LPS it detects are shared by other chlamydiae and possibly other bacteria.

It has been reported that C. abortus persists in subepithelial cells in the vagina, uterus, and oviduct of chronically infected ewes. Invasion of the chlamydiae beyond the mucosal epithelial cells provide opportunity for persistence. So it possible that viable C. abortus had not been excreted secretion during the period of collection.

The Polymerase Chain Reaction (PCR), which is easy, sensitive and quick method of detection, is now widely used for the detection of C. abortus. One advantage of using PCR for the detection of EAE is that viable EB do not have to be present, unlike the requirements for cell culture isolation. A PCR was used to detect the genome of Chlamydocila abortus in samples of uterine tissue collected from 304 sheep. The total prevalence of the chlamydial genome was 30.9 per cent, with a significantly higher prevalence in the pregnant animals as 46.9 per cent. (10)

In the present study PCR was used as a confirmatory tool after extracting DNA from faeces and vaginal secretions' using primers specific to the MOMP gene as described by Creelan and McCullaugh (4). The use of ovine abortificient strain-specific primers for the detection of DNA from placental tissues was reported to be a sensitive and specific. The specificity of clone 8 primers for C. abortus avoids the potential detection of C. pecorum, which is known to infect sheep (6). None of the samples tested were positive, suggesting that either there were no EBs ofC. abortus in the samples or there were non infectious forms (RBs) which could not be detected by DNA amplification. It has been reported that chlamydiae can be detected at early stage by ribosomal RNA (rRNA).

A variety of sample preparation methods such as immunomagnetic separation (IMS) have successfully been applied to improve the
sensitivity of PCR assays (5). Real-time PCR analysis of placental samples identified very few or no chlamydial genomes, which contrasted significantly with samples taken at the time of abortion. The results suggested that the low levels of chlamydial DNA detected during the peri-ovulation period and at lambing do not significantly impact on the epidemiology of EAE. In terms of flock management, the products of abortion should be considered the major and principal source of infection for transmission to naïve ewes. (8)

The *C. abortus* was detected by PCR for the first time in milk samples collected from ewes with EAE history using the IMS technique. However, the absence of a *C. abortus* using PCR and IMS in some animals with EAE history does not necessarily mean that they were free from the disease, as the possibility of excretion in milk is considered to be rather low (17). Chlamydiae may be present in low numbers in ewes and shed only intermittently or that other animals could act as reservoirs of infection (3). It is possible that the organism did not persist in the aborting ewes or the concentration of *C. abortus* DNA in the reproductive tract of persistently infected ewes so low, that it cannot be detected by PCR.

The peri-glandular region of basal zone of endometrium is an area where infiltrating macrophages reside (7). Infiltration of macrophages in peri glandular cells may help presentation of chlamydial antigen to be presented to MHC Class II and to stimulate the production of systemic antibodies. The serum samples from these ewes showed significantly higher chlamydia-specific IgG titre during the period of peri-ovulation. This finding is in close agreement with that of Papp et al. (11). There was also an increase in antibody reactivity during the peri-ovulation period in 3 of the 8 sheep examined, with a slight increase in IgM and IgG antibodies. Specific antichlamydial antibodies can neutralize chlamydial infectivity *in vitro* by preventing attachment to target cells or facilitating intracellular destruction. In terms of flock management, as suggested earlier by many researchers (8), the products of abortion should be considered the major and principal source of infection for transmission to naïve ewes.

References


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