Chlamydia psittaci Infection and Associated Infertility in Sheep

John R. Papp, Patricia E. Shewen and Cathy J. Gartley

ABSTRACT

Nineteen ewes were injected subcutaneously with the agent of enzootic ovine abortion, *Chlamydia psittaci* serovar 1, at 50 days gestation. Placental and fetal tissues were examined at 15 days postinfection and thereafter at ten day intervals. Placental infection was detected at 15 days postinfection. Only postinoculation sera collected from postinfected ewes contained antibodies reactive to *C. psittaci*. Five (26%) chlamydial infected ewes experienced inapparent fetal loss before day 105 of gestation. This finding is significant since *C. psittaci* infection in sheep is commonly associated with abortion and not infertility.

RÉSUMÉ

Dix-neuf brebis ont été injectées par voie sous-cutanée avec *Chlamydia psittaci* sérotype 1 au cinquantième jour de gestation. Quinze jours après l'inoculation, et par la suite aux dix jours, des tissus foetaux et placentaires ont été examinés. Une infection placentaire a été notée dès le 15e jour postinoculation et des anticorps anti *Chlamydia* ont été retrouvés seulement chez les animaux inoculés. Au 105e jour de gestation, cinq brebis infectées (26 %) avaient perdu leurs foetus sans manifestation clinique apparente. Ces résultats démontrent que les infections à *C. psittaci* chez le mouton sont associées à de l'avortement et non à de l'infertilité. (*Traduit par Dr Pascal Dubreuil*)

INTRODUCTION

Chlamydia psittaci infection of the genital tract is associated with salpingitis in guinea pigs (1) and abortion or poor fetal development in ruminants (2). Ovine abortion induced by *C. psittaci* serovar 1 is known commonly as enzootic abortion and is a major cause of worldwide reproductive failure in sheep. In Canada, enzootic abortion was shown to be responsible for 46.8% of all diagnosed ovine abortions from 1978 to 1982 (3). Pregnant sheep initially infected with *C. psittaci* either abort late in gestation or give birth to weak or stillborn lambs (2). Chlamydiae are transmitted via aerosols originating from infected placentas or tissues of aborted lambs, or from ingestion of feed contaminated by infected placentas or feces (4). The intestinal tract is a probable reservoir for the agent (2). Susceptible sheep may initially become infected by ingesting or inhaling *C. psittaci* during the previous or current lambing season. It has been suggested that infection becomes established first in the tonsil (5) from which it may disseminate by blood or lymph to other organs, where it may remain latent (4,6) with subsequent, possibly intermittent, low-grade chlamydemia. Ewes are seldom affected clinically following abortion and remain fertile, although a small proportion may develop metritis (7).

Chlamydia trachomatis infection of the reproductive tract is considered to be a major cause of infertility in women (8). There are few reports on the role of *C. psittaci* in infertility in ruminants. Artificial insemination of cows with chlamydiae contaminated semen resulted in a decreased conception rate when compared to control cows (9). Uterine biopsy samples demonstrated chlamydial multiplication in subepithelial cells of the uterine horns. Since the organism did not affect the quality of the bovine semen, it was suggested that infertility resulted from an alteration of the uterine environment. This hypothesis was supported by the observation that three day-old embryos from similarly inseminated cows did not contain any chlamydial inclusions (9). The rate of failed conception in sheep infected with *C. psittaci* is unknown. In the present report, we describe the serendipitous finding that ewes experimentally infected with *C. psittaci* at the end of their first trimester of pregnancy may experience inapparent fetal loss.

MATERIALS AND METHODS

CHLAMYDIA CULTURE AND PURIFICATION

Chlamydia psittaci isolate V287 was recovered from an aborted ovine fetus in 1988. The organism was originally isolated by inoculation of infected fetal tissue into the yolk sac of seven-day-old embryonated hens' eggs as previously described (10). HeLa 229 cell monolayers were grown in 175-cm^2 polystyrene culture flasks (Fisher Scientific, Unionville, Ontario) in 50 mL of Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (Gibco BRL, Burlington, Ontario), 100 μg of vancomycin hydrochloride per mL (Sigma Chemical Company, St. Louis, Missouri) and 25 U of nystatin per mL (Sigma). Confluent monolayers were infected with yolk sac propagated *C. psittaci* isolate V287 suspended in sucrose-phosphate-glutamic acid (SPG; pH 7.2) by shaking on a rocking platform at room temperature for four hours. Fresh EMEM containing 0.5 μg of cycloheximide per mL (Sigma) was added to the cells and the

Department of Veterinary Microbiology and Immunology (Papp, Shewen) and Department of Population Medicine (Gartley), Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1.

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flasks were incubated at 37°C and 5% CO_2 for three to four days. Infected monolayers were gently dislodged with glass beads and centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was collected and the pellet resuspended in SPG. Following brief sonication, the SPG suspension was centrifuged again as described above. The supernatants were pooled and centrifuged at 30,000 × *g* for 30 min at 4°C (50.2 Ti rotor; Beckman Instruments Inc., Mississauga, Ontario). The pellets containing infectious chlamydiae were resuspended in SPG to approximately 1/10 the original volume and stored at −135°C. Preparations were confirmed free of viruses, mycoplasmas or other bacteria by culture (11). The titer of the infectious stock was determined as previously described (10).

Chlamydial antigen required for enzyme linked immunosorbent assay (EIA) was prepared from similarly infected M^{c}Coy cell monolayers. *Chlamydia psittaci* V287 infectious stock was used to inoculate McCoy cell monolayers grown in EMEM supplemented with 10% fetal bovine serum and 200 μg streptomycin (Sigma). Following four days incubation at 37°C and 5% CO_2, chlamydiae were recovered as described above. The elementary bodies (EBs) were then partially purified by layering them over 10 mL of 35% (vol/vol) Renograffin solution (diatrizoate melgumine and diatrizoate sodium, 76% for injection; E.R. Squibb and Sons, Princeton, New Jersey) in 0.01 M Hepes (Sigma) containing 0.15 M NaCl (Fisher Scientific) and centrifuged at 43,000 × *g* for one hour at 4°C (SW27 rotor; Beckman). The pellet was resuspended in approximately 5 mL SPG and centrifuged at 30,000 × *g* for 30 minutes at 4°C. One to 2 mL of SPG was used to resuspend the pellet in each centrifuge tube and the EB suspension was stored at −135°C. The protein concentration of the partially purified chlamydial EBs (12) was determined by the method of Lowry *et al* (13).

SHEEP

Nineteen one to two year old Arcott ewes, confirmed pregnant by ultrasound examination at 40 days gestation, were obtained from a flock free of *C. psittaci* serovar 1. The sheep used for this study were housed in a disease containment facility. When the ewes reached 50 days gestation, they were injected subcutaneously above the left scapula with 1 mL of infectious *C. psittaci* V287 containing an estimated 10^7 egg-lethal-dose 50%. Rectal temperatures were recorded daily for five days pre- and ten days postinfection. All sheep were bled by jugular venipuncture at 50 days gestation and again when the pregnancy was terminated by cesarean section or abortion or when the ewes were discovered to be open.

Fifteen days postinfection, two ewes were selected *ad hoc* and sedated with xylazine (AnaSed, Lloyd Laboratories, Shenandoah, Iowa). A cesarean section was performed following localized administration of xylocaine (Lidocaine Hydrochloride, Astra Pharma Inc., Mississauga, Ontario). The fetus was exposed and approximately 3 mL of blood withdrawn from the umbilical artery. The fetus was removed and immediately euthanized by cardiac infusion with sodium pentobarbital (Euthanyl forte; M.T.C. Pharmaceuticals, Cambridge, Ontario). One or two placental cotyledons and approximately 10 mL amniotic fluid were collected in an aseptic manner. The incisions were sutured and the ewe was allowed to recover. This procedure was repeated at ten day intervals between 65 and 125 days gestation. Two ewes were selected *ad hoc* at each sampling period except for day 105 when only one ewe was selected.

The animal experimentation was in accordance with the Canadian Council on Animal Care.

Sheep that experienced inapparent fetal loss were rebred the following year. The ewes were prepared for breeding by synchronization of their oestrous cycles using vaginally applied progesterone sponges (Veramix, Upjohn Company, Orangeville, Ontario) and injectable pregnant mare's serum gonadotropin (Equinex, Ayerst Laboratories, Montreal, Quebec). The ewes were mated to a ram obtained from the chlamydiae-free source flock. Pregnancy diagnosis was completed by ultrasound examination at 40 days following introduction of the ram. Ewes that were determined to be pregnant were reinfected with *C. psittaci* V287 as before except at day 60 of gestation.

The Clearview Chlamydiae test kit (Unipath Inc., Nepean, Ontario) was used to detect the presence of chlamydiae specific lipopolysaccharide (LPS) in rectal and oral swabs taken from each fetus. Placental, vaginal and amniotic fluid swabs were also tested with the Clearview Chlamydiae kit. Chlamydiae isolation was attempted in M^{c}Coy cells from the maternal and fetal samples as previously described (14). Vaginal swabs were obtained from any animal that aborted or lambed and similarly examined for the presence of chlamydiae. Bacteriological examination of the removed tissues and vaginal swabs included plating the samples onto 5% citrated calf blood agar, MacConkey agar and Karmali agar plates. The plates were incubated 24 to 48 h in conditions described elsewhere and bacterial isolates were identified using conventional methodologies (12).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Fetal and maternal sera were tested for the presence of chlamydial-specific antibodies by an EIA. Microtitration plates (Gibco BRL) were inoculated with partially purified *C. psittaci* V287 EBs, diluted to 2 μg protein per 100 μL phosphate buffered saline (PBS), and incubated for four hours at 37°C. The plates were washed with PBS containing 0.05% Tween 20 (Fisher Scientific) and 0.5% fish skin gelatin (Fisher Scientific) warmed to 37°C. Uncoated sites in the microtitration plate wells were then blocked by incubation with PBS containing Tween 20 and fish skin gelatin (block buffer) for 30 min at 37°C in a humidified incubator. Test sera were diluted in block buffer at a concentration of 1:400 in volumes of 100 μL per well and the samples were replicated four times on each plate as described by Wright (15). The plates were incubated at 37°C for two hours in a humidified incubator and then washed three times with block buffer as before. Rabbit antisheep IgG (H and L) alkaline phosphatase conjugate (Kirkegard and Perry, Gaithersburg,

TABLE I. Comparison of antigen detection and cell culture for the identification of chlamydiae in ovine clinical samples from sheep experimentally infected with *Chlamydia psittaci* serovar 1

Day post-infection	Day gestation	Maternal samples[a]						Fetal samples[a]			
		Placenta		Amniotic fluid		Vaginal swab		Oral swab		Rectal swab	
		Antigen detection[b]	Cell culture	Antigen detection[b]	Cell culture	Antigen detection[b]	Cell culture	Antigen detection[b]	Cell culture	Antigen detection[b]	Cell culture
15	65	1[c]/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
25	75	2[c]/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
35	85	2[c]/2	0/2	0/2	0/2	0/2	0/2	0/3	0/3	0/3	0/3
45	95	2/2	2/2	1[c]/2	0/2	0/2	0/2	0/7	0/7	0/7	0/7
65	115	2/2	2/2	2/2	2/2	0/2	0/2	3/3	3/3	3/3	3/3
75	125	2/2	2/2	2/2	2/2	0/2	0/2	3/3	3/3	3/3	3/3

[a]Number of positive samples / total number of samples tested
[b]Detection of chlamydial lipopolysaccharide by the Clearview Chlamydiae kit (Unipath Inc., Nepean, Ontario)
[c]Weak positive reaction

Maryland) was added at a concentration of 1:5000 and the plates incubated for one hour as before. The plates were washed three times and color was developed by adding *p*-nitrophenylphosphate (Sigma). The positive control serum was obtained from a ewe experimentally infected with *C. psittaci* and used at a dilution of 1:400. The negative control was serum collected from an unifected ewe. The optical density (OD) at 405_{nm} was recorded with a microplate autoreader (Biotechnology Instruments, Winooski, Vermont) when the positive control gave an OD reading of approximately 1. Results were expressed as a ratio of the OD reading of the test sample to the OD reading of the positive control.

STATISTICAL ANALYSIS

Stringency of the EIA was assigned using the coefficient of variation between quadruplicate positive control wells on each plate. Data obtained from a plate were used only if the coefficient of variation was less than or equal to 5%.

The 1990 to 1992 breeding reports of the flock that supplied the sheep for this study were obtained to provide data for comparison. These data were analyzed using the Student's *t*-test (16) to determine the significance of the reproductive failure observed in the 19 ewes experimentally infected with *C. psittaci*.

RESULTS

All sheep developed a febrile response approximately 24 hours after injection with *C. psittaci* V287. An elevated temperature of one to three degrees above pre-inoculation means was recorded for up to six days postinfection. There were no other overt clinical signs associated with chlamydial infection until abortion which resulted in a slight increase in temperature (less than 3°C). Three ewes aborted their pregnancies at approximately 135 days gestation.

Chlamydiae specific antibodies were detected only in postinoculation sera and the OD values ranged from 0.195 to 1.258. Postinoculation sera obtained from ewes that lost their pregnancy were slightly less reactive to chlamydial EBs than sera from sheep that did not show any signs of fetal loss. However, this difference was not statistically significant. There was no antibody response to *C. psittaci* in any fetal sera collected.

Chlamydiae were consistently identified in maternal and fetal samples taken from 45 to 65 days postinfection (Table I). Placental swabs taken from ewes 15 to 35 days postinfection and amniotic fluid in one ewe at 45 days postinfection gave weak positive reactions using the chlamydiae LPS detection kit. Chlamydiae were not identified in vaginal swabs taken from sheep undergoing cesarean section. However, both the Clearview Chlamydiae kit and cell culture were successful in detecting chlamydiae in postpartum vaginal swabs taken from two ewes that aborted and one ewe that gave birth to a weak lamb. Other than chlamydiae, there were no significant bacterial isolates.

The flock that supplied the sheep for this study had 361 pregnancies, diagnosed by ultrasound at day 40 of gestation, over a two year period and only five failed to lamb. The remaining 356 pregnancies had successful outcomes. Five out of 19 pregnant sheep experimentally infected with *C. psittaci* at day 50 of gestation were no longer pregnant by day 105. The reproductive failure observed in the 19 experimentally infected animals was significantly higher ($p < 0.05$) than in the source flock. The gestational age at which these five sheep experienced inapparent fetal loss varied from 65 to 105 days (Table II).

The five infertile ewes were successfully rebred the following year. The sheep developed a febrile response following *C. psittaci* infection similar to the primary infection except that it lasted approximately two days. There was no evidence of fetal loss in the subsequent pregnancy.

DISCUSSION

Chlamydial infections in sheep can result in a wide variety of clinical conditions such as pneumonia, abortion, urogenital infections, mastitis, polyarthritis-polyserositis, diarrhea, encephalomyelitis, hepatitis, and conjunctivitis (2). Although the various syndromes are usually related to particular *C. psittaci* serovars, Storz (17) demonstrated that *C. psittaci* isolated from the intestinal tract of clinically normal sheep caused abortion in susceptible ewes. Therefore, the biological and pathogenic difference associated with *C. psittaci* serovars is not an absolute relationship. *Chlamydia psittaci* serovar 1 is recognized as the primary cause of enzootic ovine abortion (18) although the exact etiology of the disease has not been elucidated.

TABLE II. Summary of pregnancy diagnosis of sheep experimentally infected with *Chlamydia psittaci* serovar 1

Day postinfection	Day gestation	Number of sheep	Number of ovine laparotomies	Number of sheep examined for pregnancy by ultrasound	Number of nonpregnant ewes	Number of pregnant ewes
−10	40	19	0	19	0	19
15	65	19	2	17	1[a], 1[b]	16
25	75	16	2	14	0	14
35	85	14	2	12	1[b]	11
45	95	11	2	9	0	9
55	105	9	1	8	1[a], 1[b]	7
65	115	7	2	5	0	5
75	125	5	2	3	0	3

[a]Diagnosed not pregnant by laparotomy
[b]Diagnosed not pregnant by ultrasound

It is generally accepted that ewes not previously exposed to *C. psittaci* serovar 1 are susceptible to infection and subsequent abortion. However, the progression and clinical manifestation of the disease may vary depending on the breeding status of the animal.

Sheep experimentally challenged with *C. psittaci* serovar 1 by subcutaneous or tonsillar infection beyond day 60 of gestation either abort late in gestation or give birth to weak lambs (19,20). Buxton *et al* (21) demonstrated that placental pathology occurs at approximately 90 days gestation when pregnant sheep were experimentally infected with *C. psittaci* between 25 and 90 days gestation. There was no suggestion of fetal loss in pregnant ewes infected early in gestation. In the present study, five out of 19 pregnant ewes infected at day 50 of gestation lost their fetuses without any clinical signs. The route of challenge, subcutaneous injection, was the same and therefore the difference observed may be related to the chlamydial strain, inoculum quantity or breed of sheep.

The source flock for the sheep used in this study is routinely inspected for Johne's disease, Q-fever, maedi-visna virus, caseous lymphadenitis and *C. psittaci*. Animals that test positive to any of these organisms are immediately culled. Only rams with high quality semen are retained for breeding purposes. The rate of fetal death observed in the source flock was significantly less than in the 19 ewes experimentally infected with *C. psittaci*. The only known differences between the two groups are housing following pregnancy confirmation and chlamydial infection. Both groups were fed similar rations. Pregnant ewes not infected with *C. psittaci* have given birth to healthy lambs in the same housing facility as the experimental group (data not presented). Therefore, chlamydial infection appears to have contributed to fetal loss in the sheep used for this study. It was probable that much of the fetus was resorbed and the residual tissues expelled.

Although infertility may be one clinical manifestation of a patent *C. psittaci* infection, the sheep remain reproductively sound and can be bred the following year. These animals also appear to be immune to subsequent disease caused by *C. psittaci*.

Chlamydial infection of the placenta was detected 15 days postinfection by the Clearview Chlamydiae kit but not by cell culture. Recently, Woods and Timms (22) demonstrated that the Clearview kit was more sensitive than cell culture, 91% compared to 36% respectively, in detecting urogenital infection due to *C. psittaci* in koalas. Colonization of the placenta therefore occurred very quickly after systemic chlamydial infection.

Chlamydia psittaci serovar 1 has a predilection for the chorionic epithelium in the hilar region of the placentomes of ewes (23). Fetal lamb tissues can become infected with chlamydiae three to five days after inoculation of the ewe (24). It is possible that chlamydial infection of the maternal-fetal junction may lead to fetal death from placental insufficiency or fetal cytopathology. This may be recognized as fetal resorption if death occurs early in embryogenesis. It is conceivable that the gestational age at which the ewe acquires primary chlamydial infection determines that outcome of pregnancy. However, the pathogenesis of chlamydial induced infertility in sheep has not been established.

This report describes a strong association between *C. psittaci* infection and ovine infertility. Although the evidence is somewhat circumstantial, patent chlamydial infection in pregnant sheep may result in the loss of the pregnancy due to fetal resorption or mummification. Sheep producers may recognize these animals as infertile without realizing the potential role of exposure to *C. psittaci*. Therefore veterinarians and producers should be aware that endemic infection of a flock with *C. psittaci* may result in infertility and decreased conception rates as well as overt abortion, and further that some animals at risk of enzootic ovine abortion are not detectable by examination of vaginal swabs. Serology and reproductive history must be considered together in order to determine if an animal is susceptible to chlamydial-induced abortion. Further research examining the ovine reproductive tract at various stages of gestation and chlamydial infection may help elucidate ovine infertility and, in so doing, aid in the detection of these animals.

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