Presence of *Chlamydia abortus* in colostrum, milk, and vaginal discharge samples of sheep

Presencia de Chlamydia abortus en muestras de calostro, leche y secreción vaginal de ovejas

Presença de Chlamydia abortus em amostras de colostro, leite e secreção vaginal de ovelhas

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Abstract

Background: The main transmission route of *Chlamydia abortus* is by ingesting the microorganism that has been eliminated in vaginal secretions, placental membranes or abortions that contaminate the environment and, possibly, through milk and colostrum. Elimination through vaginal secretions is well documented. However, there are no reports about isolation and identification of *C. abortus* in the colostrum or milk of infected sheep, so it is important to determine whether or not *C. abortus* may be present in these secretions, which are the only food of lambs. **Objective:** To detect *C. abortus* in colostrum, milk, and vaginal secretions of sheep with a history of reproductive disorders. **Methods:** Colostrum, milk, and vaginal exudates were collected from 66 sheep. The samples were inoculated in mouse fibroblast cell cultures and the presence of *C. abortus* determined by direct immunofluorescence. **Results:** 19 out of 66 colostrum samples (28.7%), 14 out of 66 milk samples (21.2%) and 17 out of 66 vaginal swabs (25.7%) were positive for *C. abortus*. The 50 samples positive for isolation and detected by immunofluorescence, together with 42 negative samples were subjected to qPCR to amplify a fragment of the *omp*A gene from *C. abortus*. Thirty-eight of the 92 samples processed by this technique were positive for *C. abortus*. **Conclusion:** The results demonstrated the presence of *C. abortus* in a high proportion in colostrum, milk and vaginal secretions of infected sheep. To the best of our knowledge, this is the first field study confirming the presence of *C. abortus* in colostrum, which shows that excretion of *Chlamydia* by lactogenesis could occur in the first hours after birth.

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Keywords: *Chlamydia abortus; chlamydial infection; colostrum; ewes; milk; ovine; secretion; sheep; vaginal discharge.*

Resumen

Antecedentes: La principal vía de transmisión de *C. abortus* es la ingestión del microorganismo que ha sido eliminado en las secreciones vaginales, membranas placentarias, abortos y, posiblemente, a través de la leche y el calostro. La eliminación a través de secreciones vaginales está bien documentada. Sin embargo, no existen reportes del aislamiento e identificación de *C. abortus* en el calostro o la leche de ovejas infectadas, por lo que es importante determinar si la bacteria puede o no estar presente en estas secreciones, que son el único alimento de los corderos. **Objetivo:** Detectar la presencia de *C. abortus* in calostro, leche y secreciones vaginales de ovejas con antecedentes de problemas reproductivos. **Método:** Con el propósito de aislar e identificar *C. abortus* en estas secreciones, se recolectó calostro, leche y exudado vaginal de 66 ovejas. Las muestras fueron inoculadas en cultivos celulares de fibroblastos de ratón y se determinó la presencia de la bacteria por inmunofluorescencia directa. **Resultados:** Fueron positivas 19 de 66 muestras de calostro (28,7%), 14 de 66 muestras de leche (21,2%) y 17 de 66 hisopos vaginales (25,7%). Las 50 muestras positivas al aislamiento y detectadas por inmunofluorescencia, junto con 42 negativas se sometieron a qPCR para amplificar un fragmento del gen *omp*A de *C. abortus*; 38 de las 92 muestras procesadas por esta técnica fueron positivas para *C. abortus*. **Conclusión:** Los resultados del presente estudio demostraron la presencia de *C. abortus* en una alta proporción en el calostro, la leche y las secreciones vaginales de ovejas infectadas. Este es el primer estudio de campo que confirma la presencia de *C. abortus* en calostro, lo que demuestra que la excreción de clamidia por lactogénesis podría ocurrir en las primeras horas después del nacimiento.

Palabras clave: *calostro; Chlamydia abortus; clamidia; descarga vaginal; eliminación vaginal; infección clamidial; leche; ovejas; ovinos; secreción.*

Resumo

Antecedentes: A principal via de transmissão da *Chlamydia abortus* é a ingestão do microrganismo que foi eliminado nas secreções vaginais, membranas placentárias ou abortos que contaminam o meio ambiente e, possivelmente, através do leite e colostro. A eliminação pelas secreções vaginais está bem documentada. No entanto, não há relatos de isolamento e identificação de *C. Abortus* no colostro ou leite de ovelhas infectadas, por isso é importante verificar se a bactéria pode estar ou não presente nessas secreções, único alimento dos cordeiros. **Objetivo:** Detectar a presença de *C. Abortus* no colostro, leite e secreções vaginais de ovelhas com histórico de distúrbios reprodutivos. **Métodos:** Para isolar e identificar *C. Abortus* nessas secreções, foram coletados colostro, leite e exsudato vaginal de 66 ovelhas. As amostras foram inoculadas em cultura de células de fibroblastos de camundongo e a presença da bactéria determinada por imunofluorescência direta. **Resultados:** 19 de 66 amostras de colostro (28,7%), 14 de 66 amostras de leite (21,2%) e 17 de 66 esfregaços vaginais (25,7%) sendo positivos. As 50 amostras positivas para isolamento e detectadas por imunofluorescência, juntamente com as 42 negativas, foram submetidas a qPCR para amplificar um fragmento do gene *omp*A de *C. Abortus*. Trinta e oito das 92 amostras processadas por esta técnica foram positivas para *C. Abortus*. **Conclusão:** Os resultados do presente estudo demonstraram a presença de *C. Abortus* em alta proporção no colostro, leite e secreções vaginais de ovelhas infectadas. Este trabalho é o primeiro estudo de campo na literatura científica confirmando a presença de *C. Abortus* no colostro, o que mostra que a excreção da clamídia por lactogênese pode ocorrer nas primeiras horas após o nascimento.

Palavras-chave: *colostro; Chlamydia abortus; clamídia; corrimento vaginal; eliminação vaginal; infecção por clamídia; leite; ovelha; ovino; secreção.*

Introduction

Chlamydia affects sheep, goats and cows causing significant economic losses, and is a zoonosis (Longbottom and Coulter, 2003; Ortega *et al.*, 2016; Rojas *et al.*, 2018). *C. abortus* is the most frequently isolated microorganism in reproductive problems of small ruminants in Europe. Other species of this genus have also been described, such as *Chlamydia pecorum* and *Chlamydia psittaci* (Barati *et al.*, 2017).

C. abortus can infect non-pregnant ewes and later, when conditions are favourable for the bacteria, cause abortion in the last third of gestation (Rocchi *et al.*, 2009). Environmental conditions play an important role in abortion cases in ewes. Selim *et al.* (2020) demonstrated that the incidence of abortions increases during autumn and is more common in farms lacking lambing pens.

If the infection occurs during late gestation, it can lead to sepsis and perinatal mortality or birth of weak lambs (Papp *et al.*, 1994). The bacterium has a tropism to the trophoblastic cells of the placenta where infection establishes. From this site it spreads to the surrounding chorion, affecting nutrient transport and hormonal regulation, causing abortion (Livingstone *et al.*, 2017).

The main transmission route of *C. abortus* involves ingestion of the bacteria present in vaginal secretions, placental membranes or abortions that contaminate the environment; mainly water, feed and bedding material (Nietfeld, 2001; Wattegedra *et al.*, 2020). Both sheep and goats can excrete *C. abortus* vaginally within two weeks after delivery or abortion and it remains infectious for several weeks in the environment, which increases the spread and permanence of the microorganism in the herd (Papp *et al.*, 1994; Longbottom and Coulter, 2003; Rodolakis and Laroucau, 2015). Additionally, *Chlamydia abortus* can be transmitted through embryo transfer because it adheres to and/or penetrates the zona pellucida of *in vivo* caprine embryos after *in vitro* infection. Persistence of these bacteria after washing makes the embryo a potential means of transmission during embryo transfer from infected donor goats to healthy recipients and/or their offspring (Oseikria *et al.*, 2016).

Excretion of *C. abortus* in colostrum and milk of sheep has not been demonstrated (Nietfeld, 2001; Rodolakis and Laroucau, 2015). Therefore, the objective of this study was to assess the presence of *C. abortus* in colostrum and sheep milk from a herd with a clinical history of chlamydial infection and verify its presence in vaginal secretions.

Materials and Methods

Ethical considerations

Use of animals and experimental procedures were endorsed by the institutional subcommittee for the care of experimental animals (CICUA, by its name in Spanish), of the FMVZ, UNAM; Official letter DC-2017 / 2–5.

Animals

A 500-sheep herd with Suffolk, Dorset, Hampshire, Katahdin, and their crosses was analysed. The herd, located in the State of Morelos, Mexico, presented abortion cases in recent years. All females were seronegative to smooth brucellae and had presented cases of *C. abortus* confirmed by serology and isolation. The sampled females met the following inclusion criteria: being pregnant and in the last third of gestation; having a history of reproductive problems; and having positive serology for *Chlamydia* spp. Ewes ranged from 2 to 5 years of age, so first-parity and multiparous ewes with two and three lambings were included. The animals were grazing during the day and enclosed in pens during the night.

Sample A included a total of 66 ewes that met the inclusion criteria mentioned above. During the period from March to April 2017, vaginal swabs and colostrum were collected from these 66 ewes immediately after parturition and milk samples were collected after 30 days of lactation.

All samples (198) were stored in a sucrose-phosphate-glutamine (SPG) transport medium. The vaginal swabs were placed in 2 ml of SPG medium and kept at 2 to 8 °C during shipment to the laboratory where they were stored at −80 °C until processing. Milk and colostrum were mixed with SPG transport medium in a 1:1 ratio in 5 ml final volume and refrigerated during transport to the laboratory, where they were stored at −80 °C (Longbottom and Coulter, 2003).

Isolation in cell culture and direct immunofluorescence

Colostrum, milk and vaginal swab samples were cultured in eight-well plates (LABTEKTM Thermofisher, USA) with Dulbecco's modified Eagle's medium (DMEM, *In Vitro*, Mexico) supplemented with 10% fetal calf serum (FBS; Cellgro, USA), following the protocol described by Salinas *et al.* (1994). The mouse fibroblast cell line L929 was kept at 37 °C, with 5% CO_2 for 24 h. When the monolayer presented from 70 to 80% confluence the medium was removed and the cells were inoculated with 100 µl of the samples in each well of the plate. Subsequently, they were incubated for 5 min at 37 °C in an incubator with an orbital shaker to 50 g. They were then centrifuged at 730 g for 15 min and incubated in 5% CO_2 at 37 °C for 1 h. Next, the inoculum was removed, 300 µl of the supplemented medium was added, and the plate was incubated again under the same conditions for up to 72 h. The medium was discarded from the plates, washed with sterile 1X Phosphate-Buffered Saline (PBS) and the wash solution was removed and the plate fixed in cold methanol for 15 min at 4 °C. The fixative solution was subsequently removed and washed again with 1X PBS. Direct immunofluorescence test (DIF) was performed with a commercial kit designed to detect chlamydial lipopolysaccharide (LPS). However, this test does not differentiate between species of the *Chlamydiaceae* family (ImageTM *Chlamydia* OXOID Kit, UK). The procedure was carried out following the manufacturer's instructions. The slides were observed with a fluorescence microscope (Leica DM 6000B, Germany) to detect the presence of chlamydial inclusions in cultures previously processed with colostrum, milk and vaginal swab samples.

Real-time PCR (qPCR)

For the qPCR test, DNA from colostrum, milk, and vaginal secretion samples was included. Therefore, a total of 92 samples were processed using qPCR, including 50 positive samples (18 for colostrum, 14 for milk, and 18 for vaginal discharge), as well as 42 negative samples (13 for colostrum, 16 for milk, and 13 for vaginal discharge). The DNA from the vaginal swabs was extracted using a commercial kit (Qiagen DNeasy Blood and Tissue, Germany) following the protocol described by the manufacturer. A modification of the phenol-water technique was used for DNA extraction for colostrum and milk samples, as described by Sambrook and Fritsch (1989). The samples were thawed at 37 °C for 30 min, then 300 µl of colostrum or milk were taken, and 5 µl of lysozyme and 500 µl of solution 1 (1 M Tris HCl, pH 8; 0.5 M EDTA, pH 8) were added, homogenised in a mixer (Vortex Genie-2, Scientific Industries, Canada) and incubated overnight at 56 °C. The following day, 100 µl of STEP solution (0.5% SDS, 50 mM Tris HCl and 0.5 M EDTA pH 8) and 1.5 µl of proteinase K were added. The tubes were carefully shaken and incubated for 1 hr, 37 °C with occasional stirring. Subsequently, a volume (1–1.5 ml) of buffered phenol was added and vigorously shaken to emulsify and centrifuge at 1,372 *g* for 15 min. The aqueous phase was recovered in a sterile 2 ml tube, one-tenth of its volume of potassium acetate was added and mixed carefully, then two volumes of cold ethanol were added and again it was carefully shaken. The sample was centrifuged at 11,200 *g* for 30 min. Finally, the ethanol was decanted and allowed to dry overnight at room temperature. DNA was resuspended in 10:1 Tris EDTA (TE) buffer, quantified and stored at −20 °C until use.

For the qPCR test, a fragment of the *omp*A gene from *C. abortus* was amplified. qPCR was performed using primers and probes previously described by Pantchev *et al.* (2009). The primers and probes were CpaOMP1-F:

GCAACTGACACTAAGTCGGCTACA, CpaOMP1-R: ACAAGCATGTTCAATCGATA TAAGAGA, CpaOM P1-S: FAM-TAAATACC A C G A A T G G C A A G T T G T T T A G C G-TAMRA, respectively. The concentration was 0.9 µM with a 0.2 µM probe. The amplification conditions were initial denaturation at 95 °C/5 min, denaturation at 95 °C/3 s and an extension of 60 °C/30 s, in a total of 40 cycles. DNA from *C. abortus* strain AB7 was used as a positive control.

Statistical analysis

Cohen's kappa coefficient (κ) was used to determine the degree of agreement between tests.

$$k = \frac{p_o - p_e}{1 - p_e}$$

Where:

p_o is the relative observed agreement among raters

p_e is the hypothetical probability of chance agreement.

Results

Isolations and DIF

Of the 198 samples inoculated in cell culture and evaluated with the DIF technique (66 colostrum samples, 66 milk samples and 66 vaginal swab samples), a total of 50/198 (25.25%) were positive for the presence of chlamydial antigen, of which 19/66 (28.7%) were from colostrum, 14/66 (21.21%) from milk, and 17/66 (25.7%) from vaginal swabs (Table 1).

Table 1. *Chlamydia* spp. positive samples by isolation and direct DIF.

	Positive	%	Total
Colostrum	19/66	28.7%	66
Milk	14/66	21.2%	66
Vaginal swab	17/66	25.7%	66
Total	50/198	25.3%	198

Of the 50 positive samples, 18 sheep presented at least two positive samples to isolation and DIF; 12 animals were positive for both colostrum and vaginal swab, while nine sheep were positive for vaginal swab and milk. Regarding the colostrum–milk relationship, only eight sheep tested positive in both samples. Overall, five sheep had all three negative samples and four sheep had all three positive samples (colostrum, milk and vaginal swab) for isolation by culture and DIF.

Results obtained in real-time PCR (qPCR)

A total of 92 samples were processed by the qPCR technique, 50 positives for culture and DIF, and 42 negatives. Results indicate that 38/92 samples (41.3%) were positive for qPCR, of which 16 were from colostrum, four from milk, and 18 from vaginal secretions. Derived from the results, it was possible to establish the kappa (κ) concordance index between isolate/DIF and qPCR for the different sample types (Tables 2 to 4).

Table 2. Concordance between isolation and qPCR in colostrum samples for detection of *Chlamydia* spp.

		Isolation/DIF		
		Positive	**Negative**	**Total**
qPCR	**Positive**	15(48.3%)	1(3.2%)	16(51.6%)
	Negative	4(12.9%)	11(35.4%)	15(48.3%)
	Total	19(61.2%)	12(38.7%)	31(100%)
Kappa index for colostrum = 0.81			Colostrum: n=31	

Interpretation: <0.00=no agreement; >0.00–0.20=negligible; 0.21–0.40=discrete; >0.41–0.60=moderate; 0.61–0.80=substantial; 0.81–1.00=almost perfect.

Table 3. Concordance between isolation and qPCR in milk samples for detection of *Chlamydia* spp.

		Isolation/DIF		
		Positive	**Negative**	**Total**
qPCR	**Positive**	4(13.3%)	0(0.0%)	4(13.3%)
	Negative	10(33.3%)	16(53.3%)	26(86.6%)
	Total	14(46.6%)	16(53.3%)	30(100%)
Kappa index for milk=0.14			Milk: n=30	

Interpretation: < 0.00 = no agreement; >0.00–0.20=negligible; 0.21–0.40=discrete; >0.41–0.60=moderate; 0.61–0.80=substantial; 0.81–1.00=almost perfect.

Table 4. Concordance between isolation and qPCR in vaginal discharge samples for detection of *Chlamydia* spp.

		Isolation/DIF		
		Positive	**Negative**	**Total**
qPCR	**Positive**	12(38.7%)	6(51.6%)	18(58.0%)
	Negative	5(16.1%)	8(25.8%)	13(41.9%)
	Total	17(54.8%)	14(77.4%)	31(100%)
Kappa index for vaginal secretions = 0.50 discharge: n=31		Vaginal	Milk: n=30	

Interpretation: <0.00=no agreement; >0.00–0.20=negligible; 0.21–0.40=discrete; >0.41–0.60=moderate; 0.61–0.80=substantial; 0.81–1.00=almost perfect.

The observed concordances were 0.81 (almost perfect), 0.14 (insignificant) and 0.50 (moderate) for colostrum, milk, and vaginal secretions, respectively.

Of the 42 samples negative for the fluorescence test, only one colostrum and six vaginal swabs were positive for qPCR. Of the 18 females that tested positive for qPCR in the vaginal swab sample, 10 (55%) were also positive in colostrum, while only three positive samples of milk (16.6%) were from three of the 18 sheep that tested positive in vaginal swabs for the same test.

Regarding the colostrum–milk relationship, only two milks of the 16 positive colostrum samples (12.5%) by qPCR, were from the same sheep.

Overall, only one sheep had three positive samples and two sheep had all three negative samples (colostrum, milk, and vaginal discharge) for both qPCR and isolation by culture and DIF.

Discussion

In the present study, the presence of *C. abortus* in colostrum, milk and vaginal secretions of infected sheep was evaluated, with a specific interest in demonstrating presence of this bacterium in colostrum and milk.

Rodolakis and Laroucau (2015) indicated that the percentage of abortions caused by *C. abortus* in sheep herds in Europe can reach up to 30% when the disease occurs for the first time in the herd, generating large economic losses. This is aggravated by stillborn lambs and weak offspring, which do not survive more than a week. Furthermore, milk production tends to decrease in affected sheep during an outbreak of the disease. In these herds, it is important to limit the spread of infection to other animals.

The main sources of infection described were vaginal secretions, placental membranes and dead foetuses, which were infected with a large number of *Chlamydia* that contaminated food, water and bedding at the time of delivery or abortion or during heat, favouring the transmission and permanence of the bacteria in affected herds (Longbottom and Coulter, 2003; Stuen and Longbottom, 2011; Rodolakis and Laroucau, 2015). If lambs ingest contaminated colostrum or milk, the bacteria could remain inactive in the animal until conditions are favourable for the disease to manifest, given the survival characteristics of *C. abortus* (Elwell *et al.*, 2016).

Infected sheep that shed *Chlamydia* in colostrum or milk could be a source of infection for lambs, since it is the only food for new-borns that are also born agammaglobulinaemic with an immature immune system that slowly produce immunoglobulins during the first hours of life (Castro *et al.*, 2011). Under these conditions, a safe way to prevent infection transmission would be to isolate new-born lambs, not allowing them to consume colostrum or milk from their mothers and to feed them milk replacement. However, milk -as well as colostrum- is the fundamental food for offspring survival so, like colostrum, it can be replaced with healthy sheep's milk or it can be pasteurised, but risking to lose part of its biological properties. (Escuder *et al.*, 2018; 2021).

Thomas *et al.* (1990) detected the presence of chlamydial antigen in samples of placenta and sheep's milk using IDEIA-ELISA. However, only one of 26 milk samples was positive. In their study, isolation was attempted in MC Coy cells. In an attempt to isolate *Chlamydia* from

the collected samples, they separated fat from milk by centrifugation and the samples were stored in the transport medium provided by the commercial kit. All samples were negative on isolation.

In the present study, cultures of the mouse fibroblast cell line L929 were used for vaginal swab, milk and colostrum samples. In addition, at the time of collection colostrum and milk were placed in SPG medium, while they were transferred to the laboratory at 4 °C and then frozen at −80 °C, which could favour bacteria viability. Before inoculating colostrum and milk in the cell cultures, they were heated to 37 °C for 30 min to favour fat dissolution, but colostrum samples were not centrifuged to separate the fat fraction. Under this procedure, the isolates of *Chlamydia* spp. were obtained in 19 colostrum and 14 milk samples by infecting cell cultures that were positive for DIF.

Analysis of kappa indices showed that the highest agreement was for colostrum/qPCR ratio (0.81), followed by vaginal secretion/qPCR (0.50) and negligible for milk/qPCR (0.14). As qPCR is a high sensitivity and specificity test, results indicate that the presence of *C. abortus* is high, especially in colostrum, representing an important risk factor for lambs as a source of infection. Similarly, these results would make vaginal discharge a second risk factor as a source of infection, and milk -after 30 days of lactation- as a minimal risk.

In relation to the lower number of positive results obtained by qPCR with respect to isolation and confirmation by DIF, the difference could be explained by the fact that the primers used were specific for *C. abortus* and the DIF may be detecting other species of *Chlamydia* that could have been recognised by DIF.

Pantchev *et al.* (2009) determined qPCR sensitivity by means of decimal dilutions of purified cell culture DNA, which contained defined numbers of Inclusion Forming Units (IFU) of C. psittaci and *C. abortus*, being able to detect up to two IFU of each pathogen per mixture reaction in qPCR, which makes it very sensitive and specific.

To the best of our knowledge, this is the first time the presence of *C. abortus* in colostrum and milk is demonstrated, which implies its possible lactogenic excretion in the first hours after birth, as found in colostrum. Similarly, it opens the possibility to consider colostrum as an important source of infection.

In conclusion, our results demonstrated the presence of *C. abortus* in a high proportion in colostrum, milk, and vaginal secretions of infected sheep. The techniques used included isolation in cell cultures and DIF, and were confirmed with a specific qPCR of *C. abortus* species.

This work is the first field study in the scientific literature confirming the presence of *C. abortus* in colostrum, which shows that excretion of *Chlamydia* by lactogenesis could occur in the first hours after birth. However, it would be necessary to carry out further studies to show whether the amount excreted is sufficient to cause infection and induce clinical symptoms during adulthood.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest whit regard to the work presented in this report.

Author contributions

María Guadalupe Martínez Serrano conducted the study, including the experimental

design, paper review and edition. Gabriela Palomares Reséndiz revised the paper and helped with the experimental design. Jorge Luis Tórtora Pérez revised the paper. Hugo Ramírez Álvarez revised the paper. Nieves Ortega Hernández helped with the training and assembly of analytical techniques. Jesús Salinas Lorente helped with the training of analytical techniques and revised the paper. José Francisco Morales Alvarez reviewed and edited the paper. Julio César Cervantes Morali conducted the animal study. Efrén Díaz-Aparicio managed the project and revised the paper.

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