

Isolation of *Chlamydia abortus* in dairy goat herds and its relation to abortion in Guanajuato, Mexico

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Abstract

Although *Chlamydia abortus* is classified as an exotic agent in Mexico, there is increasing evidence of its presence. The objective of this study was to isolate *C. abortus* in dairy goat herds with problems of abortion in the state of Guanajuato, Mexico, and to develop appropriate diagnostic methods for its detection. Serological samples and vaginal swabs were taken from 6 dairy goat herds. The ELISA revealed a seropositivity of 9.60% for *C. abortus*. The PCR test based on the vaginal mucus samples resulted in 30 of 126 positive animals (23.8%). *Chlamydia* spp. were isolated in 34 of the 126 animals tested (26.98%). The 3 diagnostic methods tested were valuable and complementary in zones where *Chlamydia* is suspected to cause abortions. We demonstrated that the bacteria are present in dairy goat herds of Mexico; thus, Veterinary Sanitary Authorities should consider this disease endemic to establish sanitary procedures to control the spread of the disease and to prevent human transmission.

Keywords: Goats; *Chlamydia abortus*; Abortion; Mexico

Introduction

Enzootic abortion in small ruminants (EASR) is an infectious disease caused by *Chlamydia abortus*, previously named *C psittaci* type I or *Chlamydophila abortus* (Andersen, 1991; Everett *et al.*, 1999), that affects sheep, goats, and cattle, provoking abortions during the final trimester of gestation or the birth of weak offspring that generally die during the first days of life (Kuo *et al.*, 2011; Longbottom and Coulter, 2003; Rodolakis, 2001; Chisu *et al.*, 2013).

Transmission among animals occurs primarily after parturition or abortion, due to the large quantity of bacteria that are spread through vaginal discharges, the placenta, and the skin of aborted fetuses (Longbottom and Coulter, 2003; Rodolakis, 2001; Gutierrez *et al.*, 2011). In Mexico, this disease is considered exotic and is therefore included in Group 1 of the agreement that lists and classifies the diseases and plagues in animals as exotic and endemic (*i.e.*, requiring obligatory notification) (SAGARPA, 2007). Despite this requirement, several reports of this disease have been made among small ruminants in Mexico. In 1996, *C. psittaci* was isolated

from flocks of sheep in 5 states (Escalante-Ochoa *et al.*, 1996), whereas, in 1997, the first reported presence of *C. psittaci* appeared in goat herds (Escalante-Ochoa *et al.*, 1997). Later, additional studies of this disease in goats were conducted. In 2005, the presence of the *Chlamydia* spp. was confirmed in the state of Michoacán, where it was successfully isolated from feces, aborted fetuses, stillbirths, and kids dead within 5 days of age (Lazcano, 2006; Lazcano *et al.*, 2005). In 2008, a serological study was conducted in dairy goat herds in 6 states of the country and antibodies against the bacteria were found (Mora *et al.*, 2008). In 2001, *Chlamydia* spp. were connected with zoonotic infections in Mexico, which were related to *Chlamydia* spp. infected goats and sheep (Escalante *et al.*, 2001; Barbosa Mireles *et al.*, 2013).

Because EASR is considered an exotic disease in Mexico, it is difficult to obtain reagents, antibodies, and diagnostic techniques that allow a faster identification in possible cases in which *C. abortus* is suspected. Moreover, the process used to isolate the agent is complex, as it requires both specialized training of the technical personnel and a live biological medium for its culture, such as chicken embryos or cell culture. Finally, the procedure for achieving isolation and identification may require several weeks (Biberstein and Hirsh, 2004; Longbottom and Coulter, 2003).

Thus, the objective of this study was to determine the presence of *C. abortus* through isolation, PCR and ELISA in dairy goats with problems of abortion that suggest Chlamydiosis in the state of Guanajuato, Mexico, and to establish appropriate diagnostic methods for its detection.

Materials and methods

Animals

Six dairy goat herds from Guanajuato, Mexico, with a history of abortion were selected. The production systems on these ranches were based on intensive stabling for cheese production and rebreeding.

To obtain the necessary information, two questionnaires were given to the owners of each herd. The first questionnaire focused on several aspects of farm management, such as genetics, nutrition, overall animal health, reproduction and facilities. The second questionnaire sought information on the individual goats sampled: age, parity, clinical history and production (Mora, 2011). The information reported in both questionnaires led us to establish the differential diagnosis for the abortion problem within each herd. The number of animals tested from each flock is shown in Table 1.

Clinical samples

A total of 126 samples of vaginal swabs were analyzed using cell culture and PCR to isolate and identify *C. abortus*; 125 serum samples from the same animals were used to detect antibodies against this microorganism by ELISA (Table 1). The goats selected for sampling fulfilled one of the following conditions: one or more previous births; at most 2 weeks prior to giving birth; a maximum of 4 weeks after a recent birth; or already had an abortion.

Table 1. Number of animals tested per flock (A-F) and number of positive samples obtained with 3 different tests.

Test	Positive samples / Number of animals tested per flock						Total number of positive samples
	A	B	C	D	E	F	
ELISA, n = 125	0/12	10/34	1/30	1/15	0/13	0/21	12
PCR, n = 126	2/12	12/34	7/31	2/15	3/13	4/21	30
Bacterial Isolation, n = 126	1/12	15/34	7/31	3/15	4/13	4/21	34

n = total number of samples

The vaginal samples were taken using sterile swabs and were transported in tubes with 2 ml of sucrose-phosphate/glutamate medium (SPG) (217 mM sucrose, 4 mM KH₂PO₄, 7 mM K₂HPO₄, and 1% L-Glutamine), supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin, 50 µg/ml gentamicin) (Sachse *et al.*, 2009). The swabs were first pressed against the walls of the tubes in which they were held, using a sterile clamp and were then discarded. Afterwards, the tubes were centrifuged at 3 000×g for 40 min at 4°C; then, 500 µl of the supernatant was extracted and transferred to a sterile microtube that was labeled and frozen at a temperature of -70°C to perform the isolation procedure. The remaining contents of the tube were transferred to another sterile microtube and frozen at -70°C for later DNA extraction.

Serological tests

The commercial kit "IDEXX Chlamydiosis Verification Test" (former "Pourquier® ELISA Chlamydiosis Serum Verification", IDEXX laboratories Inc. Westbrook, Maine, US) was used to detect a recombinant antigen that is a polymorphic external membrane protein of 80-90 kDa and that is specific for *C. abortus* and has no cross-reaction with *Chlamydia pecorum*.

The Rose Bengal test (3%) was conducted (Aba test card at 3%, PRONABIVE, DF, Mexico) to assure that there was no presence of Brucellosis in the herds.

Isolation and identification of *Chlamydia spp.*

Cellular monolayers of the L929 fibroblast were cultivated in Eagle's minimal essential medium (MEM, GIBCO, Life Technologies, Carlsbad, CA, USA), supplemented with 10% FBS, 1% non-essential amino acids, L-glutamine at 1%, and antibiotics (50 µg/ml gentamicin and 100 µg/ml streptomycin-penicillin) (MEM-C), all from Life Technologies, in humid conditions at 37°C with 5% CO₂ (Escalante-Ochoa *et al.*, 1996). For infection, the culture was conducted in 24-well polystyrene plates (NUNC™ Thermo Scientific, Waltham, MA, USA) with 12-mm diameter sterile coverslips for the immunofluorescence test and at an initial concentration of 5 × 10⁴ cells/well and an incubation period of 24 h until a confluence of 60-70% was obtained. Parallel to this, dishes without coverslips were prepared for use in case the performance of the blind passages proved necessary.

Infection process

To achieve cellular infection, the MEM-C of each well was removed and 100 μ l of the supernatant from the clinical samples was added immediately to each well. Two wells/dish were used for each clinical sample for both diagnosis and the subsequent blind passages. Each dish had a positive control sample infected with a strain of *C. abortus* A22 and an uninfected negative control. The microplates were placed in an orbital incubator at 50 rpm for 1 h at 37°C in humid conditions. Afterward, 900 μ l of MEM-C were added to each well, and the dishes were then incubated in humid conditions at 37°C with 5% CO₂ for 72 h.

At the end of the incubation procedure, the plates without coverslips were stored at -70°C; the plates with coverslips had the MEM-C removed and were washed 3 times with a phosphate saline solution (PBS), for 5 min on each occasion. Next, the PBS was removed and the cell monolayers were fixed with 1 ml of pure methanol at -20°C for 10 min. The methanol was then eliminated, and the plates were left to dry at room temperature.

Direct immunofluorescence technique

Identification of the intracytoplasmatic inclusions produced by *Chlamydia* spp. was performed by direct immunofluorescence (IMAGEN™ *Chlamydia* DakoCytomation LTD, Cambs, UK), which detects the lipopolysaccharides of the bacteria using specific monoclonal antibodies marked with fluorescein. Next, 25 μ l of fluorescein-5-isothiocyanate (FITC) diluted to 1/10 with PBS and 2 μ l of Evans Blue (0.5%) were placed on each coverslip on the microplates, followed by incubation in a humid atmosphere for 30 min at 37°C. Three washings were then performed using PBS for 5 min each, and the coverslips were removed from each well and the contents were allowed to dry at room temperature (Vanrompay *et al.*, 1994).

The coverslips were mounted on slides using Vectashield® medium (Vector Laboratories, Inc, Burlingame, CA, USA) and fixed with transparent nail polish. The preparations were analyzed using a Leica DM1000 fluorescence microscope (magnification 40X). If no cytoplasmatic inclusions were visualized on the first reading, then blind passages were performed using the plates without coverslips, previously stored at -70°C. These plates were frozen and thawed 5 times to lyse the cells and release the bacteria. The content was then transferred to sterile microtubes and new cell monolayers were infected. The samples were considered negative if after conducting two blind passages, no intracytoplasmatic inclusions were detected.

Identification of *C. abortus* by PCR

The DNA was extracted from L929 mouse fibroblast cells infected with *C. abortus* A22. In this procedure, 200 μ l of an infected cell culture was collected and deactivated in a hot water bath at 80°C for 20 min.

The DNA extraction from clinical samples was conducted via the phenol-chloroform method using 500 μ l of the transport medium with the vaginal smears, following the protocol described by Sambrook and Russell (Sambrook and Russell, 2001).

Following the same procedure, DNA was extracted from uninfected L929 mouse fibroblast cells and utilized as a negative control in PCR.

Primers

Primers used for the identification of *C. abortus* were obtained from the 16S RNAr gene with the IDT SciTools Primer QuestSM program, which amplifies 342 bp as follows: Forward (5'-TGAGGCTGATGACTGGATGAAGT-3') and Reverse (5'-GTCAATGCCAAGGCATCCACCAAT-3').

The PCR was performed in a Thermo Hybaid PCR Express thermocycler. All of the reactions were conducted using a final volume of 50 μ l that contained 1X of PCR buffer, 3 mM MgCl₂, 400 μ M dNTP, 25 pmol of each primer, 1 U of Taq Polymerase (Invitrogen, Life Technologies) and 25 ng of DNA from the cell culture infected with *C. abortus* strain A22 that was utilized as a control or 25 ng of DNA from the L929 line mouse fibroblast cells.

In addition, the DNA from different bacteria that might be involved in the infections of the goats was used including *Brucella abortus*, *Leptospira* Hardjo, *Histophilus somni*, *Salmonella* Typhi, *Campylobacter jejuni*, *Campylobacter fetus*, and *Mycoplasma bovis*. The program to amplify *C. abortus* using 16S RNAr gene primers was as follows: after an initial denaturalization period of 5 min at 95°C, the reactions were exposed to 40 cycles of 1-min at 95°C, followed by 30 sec at 63°C, then at 72°C for 1 min and a final extension step at 72°C for 10 min.

The products of the amplification procedure were observed in a 1% agarose gel supplemented with ethidium bromide (0.5 μ g/ml) (Sambrook and Russell, 2001) and analyzed in a photodocumenter (Kodak, Gel Logic200, Rochester, New York, USA) supported by Kodak Molecular Imaging Software v. 4.0.2.

Statistical analysis

The PCR used in the study was evaluated using a current concordance table that considers 3 parameters: sensitivity, specificity, and the predictive value of the test (positive and negative). The PCR validity was also determined (Armijo, 1994; Greenberg *et al.*, 2002). In order to establish the degree of concordance between diagnostic tests, the Kappa coefficient was calculated (Cohen, 1960).

Results

Serological test and bacterial isolation

The 125 sera from the goats were negative for Brucellosis. However, for the *C. abortus* tested by ELISA, 12 of the 125 goats proved positive (9.60%). The results of the processes of isolating and identifying *Chlamydia spp.* in cell culture and by direct cell immunofluorescence revealed that 34 of the 125 animals were positive (26.98%, Table 2).

PCR

The PCR did not amplify the DNA of *L. Hardjo*, *H. somni*, *S. Typhi* and *M. bovis*; however, amplification of the DNA of *B. abortus*, *C. jejuni*, and *C. fetus* was observed. Despite this finding, the identification of *C. abortus* was not impeded because the amplicons were approximately 900 bp for *B. abortus*, 400 bp for *C. jejuni*, and 800 bp for *C. fetus*.

Table 2. A comparison of 3 diagnostic tests for small ruminant enzootic abortion. The PCR and bacterial isolation were performed from vaginal swabs of goats that had recently given birth or from aborted goats.

The ELISA was conducted with the commercial IDEXX Chlamydiosis Verification Test kit (IDDEX Laboratories Inc.).

Number of goats	Isolation (n=126)	PCR (n=126)	ELISA (n=125)
7	+	+	+
17	+	+	-
1	+	-	+
2	-	+	+
9	+	-	-
5	-	+	-
2	-	-	+
83	-	-	-
126	34	30	12

Using DNA from the vaginal mucus, testing using PCR resulted in 30 positive animal tests (23.8%) (Table 2), which demonstrated a sensitivity of 70.58% and a specificity of 93.47% (using isolation in cell culture as the reference test). The positive predictive value of PCR was 80%, and the negative predictive value was 89.58%. Thus, this test achieved a validity of 87.30%. As Table 2 shows, only 7 goats tested positive when ELISA, bacterial isolation, and PCR were used.

The degree of concordance between diagnostic tests using the Kappa coefficient was as follows (Landis and Koch, 1977):

- Bacteriological isolation (the gold test) and the PCR test = 0.91 (very high)
- Bacteriological isolation and the ELISA test = 0.80 (high)
- ELISA and PCR tests = 0.20 (low)

Discussion

In Mexico, Chlamydiosis in goats caused by *C. abortus* is considered an exotic disease by the sanitary authorities (SAGARPA, 2007); however, evidence of its presence is becoming increasingly common. This study proved (through isolation and identification of the microorganism) the presence of *C. abortus* in the investigated herds, strongly suggesting that the abortions that occurred shortly before sampling could have been caused by *C. abortus*. To date, only a few isolations of *Chlamydia* have been reported, only serological and molecular proof exist of *C. abortus* presence, and there is no evidence of disease caused by *C. pecorum* (Mora-Díaz *et al.*, 2009; Aguilar *et al.*, 2011; Campos-Hernandez *et al.*, 2014).

In 2007, a total of 1105 goat sera from several Mexican states where the goat-raising industry has developed (Tlaxcala, Estado de México, San Luis Potosí, Guanajuato, and Querétaro) were analyzed using the IDEXX Chlamydiosis Verification Test (IDDEX Laboratories Inc.). From these sera, a global seropositivity of 3.17% for *C. abortus* with a variation of 0-to-24% was found among the flocks

studied (Mora-Díaz *et al.*, 2009). In 2010 and 2011, the same ELISA test was used in a study conducted in six regions that are considered Mexico's main goat-raising zones: Puebla, Guerrero, Baja California Sur, Comarca Lagunera, Tlaxcala, and San Luis Potosí and different positivity percentages of 0.18%, 4%, 5%, 7.3%, 10%, and finally 11%, respectively, were found. In that study, researchers collected samples randomly from goats older than 2 years that were raised in production units with a history of abortions (Aguilar *et al.*, 2011). Campos-Hernandez *et al.* (2014) demonstrated a high seroprevalence and molecular identification of *C. abortus* in commercial dairy goat farms in a tropical region in Mexico, although no isolation of the microorganisms has been achieved. Their results, together with those from the present study, clearly indicate that EASR is indeed present in Mexico.

However, because this is considered an exotic disease, there are still no approved diagnostic tests in Mexico that can be utilized routinely in diagnostic laboratories; a fact that makes detecting this disease even more complicated. It is important to have standardized tests that can be used quickly whenever suspicions of the existence of such exotic diseases emerge. This study demonstrated that PCR is an effective tool for demonstrating the presence of this disease in a region that is considered at risk. However, it is important to use various techniques during the process of diagnosing this disease because, while ELISA is a highly sensitive test, it is not indicative of the presence of disease but only of exposure to the etiologic agent. Thus, the 3 diagnostic methods tested are valuable and complementary in zones where *Chlamydia* is suspected to cause abortions.

The decision to work with the 6 dairy goat herds from the state of Guanajuato was made because their owners mentioned that they had experienced problems with abortions in recent years during the final trimester of gestation and had experienced births of weak offspring that died shortly after parturition. This occurred even though they are all Brucellosis free; Brucellosis is an endemic disease that can cause abortions.

It should be stressed that of the 7 animals that tested positive for all of the tests included in this study, 2 had aborted one month before the samples were taken. This indicates that even one month after aborting they continue shedding bacteria through the vagina.

It is of the utmost importance to emphasize that the herds included in this study contain animals of high genetic value whose reproductive potential is diminished by infections with *C. abortus*. We estimate that in these herds, the losses caused by the presence of an abortion are approximately \$300.00 US dollars. Furthermore, these same production units sell breeding stock to replace those used in other goat-raising regions in Mexico. Thus, it is important to implement a program designed to control this disease, and this program should be based on a combination of accessible diagnostic tests.

Conclusions

Although other agents known to cause abortions in small ruminants are considered exotic in Mexico and therefore are difficult to investigate due to lack of reagents, we demonstrated that *C. abortus* is present in Mexican dairy goat herds as determined by serological and molecular tests and finally by bacterial isolation. Even though

the best concordance in the Kappa test was between bacterial isolation and the PCR technique, we consider that all 3 tests were complementary. Thus, Veterinary Sanitary Authorities should consider this disease endemic to establish sanitary procedures to control the spread of the disease and to prevent human transmission.

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Conflicts of interest

Francisco Suárez Güemes is Secretary of Research, Innovation and Technological Development at Facultad de Medicina Veterinaria y Zootecnia. The other authors declare that they have no conflicts of interest.

Author contributions

Juan Carlos Mora Díaz: Conducted the experimental work and wrote the manuscript.
Efrén Díaz Aparicio: Designed the research and wrote the manuscript.
Enrique Herrera López and Susana Jaimes Villareal: Conducted the experimental work.
Francisco Suárez Güemes and Cristina Escalante Ochoa: Analyzed the information.
Beatriz Arellano Reynoso: Headed the research and wrote the manuscript.

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