Prevalence and molecular identification of Chlamydia abortus *in commercial dairy goat farms in a hot region in Mexico*

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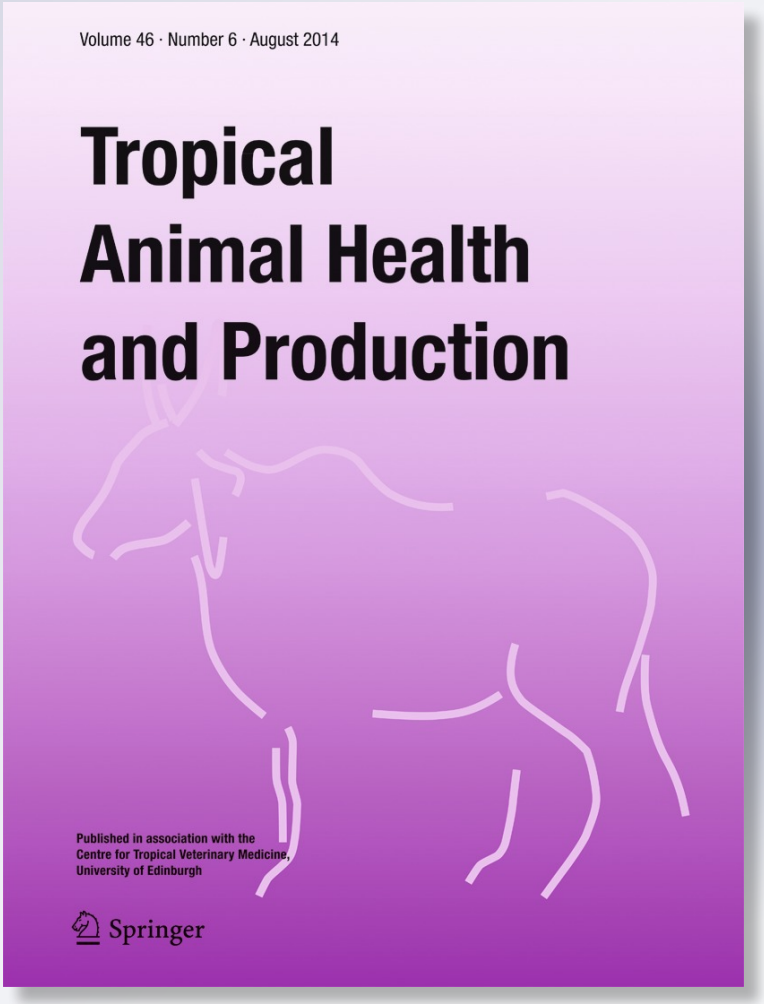

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REGULAR ARTICLES

Prevalence and molecular identification of *Chlamydia abortus* in commercial dairy goat farms in a hot region in Mexico

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Abstract The aim of this study was to determine the seroprevalence and presence of *Chlamydia abortus* in Saanen breed female goats from commercial dairy goat farms under intensive production in the municipality of Guanajuato, Mexico. Sera were collected to determine the prevalence of anti-*C. abortus* IgG antibodies using recombinant enzyme-linked immunosorbent assay (rELISA) and cell culture. Polymerase chain reaction (PCR) was used to prove the presence of the pathogen in swab samples collected from the vagina and rectum of selected animals. Additionally, foetal tissue samples from a sudden abortion were collected. *C. abortus* prevalence in female goats of commercial milking farms sampled in Guanajuato, Mexico, was 4.87 % (n=246). Seropositive animals were found in six out of nine (66.6 %) dairy goat farms sampled, and prevalence among animals in individual farms ranged between 3.44 and 13.51 %. *C. abortus* was detected using PCR in spleen tissue from the aborted foetus. PCR-based detection, as well as isolation from vaginal and rectal swabs, was not possible in the present study. Isolation through cell culture was also unsuccessful from aborted foetal tissue samples. In conclusion, the results from rELISA and PCR show that *C. abortus* is present in dairy goat farms in the state of Guanajuato, Mexico.

Keywords Prevalence · *Chlamydia abortus* · Isolation · PCR

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Introduction

Chlamydia abortus is the etiological agent of enzootic caprine and ovine abortion (Huang et al. 2013). The target organ for *C. abortus* is the placenta; therefore, infected pregnant does either abort late in gestation or give birth to weak kids (Longbottom et al. 2013). *C. abortus* infection in humans has also been reported as a public health problem because it can also result in abortion (Psarrakos et al. 2011). Additionally, it has been associated with other human health disorders, such as sepsis during pregnancy and pelvic inflammatory disease (Rodolakis and Yousef 2010; Walder et al. 2005; Wheelhouse and Longbottom 2012). A study conducted in 2002 demonstrated that people living in close contact with domestic animals are more likely to contract chlamydiosis. In Germany, it was found that there was an infection incidence of 100 cases per year, originating from human contact with enzootic abortion products (Sachse et al. 2002). Therefore, it is important to identify the foci of infection so that health authorities can take steps towards prevention and control of the infection in humans. From an economical and sanitary point of view, it is widely accepted that *C. abortus* is an important pathogen for ruminants that is widely distributed around the world, even in highly developed countries (van den Brom et al. 2012). For instance, in a study conducted in Switzerland with sheep, abortion prevalence was estimated at 19 % (Borel et al. 2004). Another study conducted in Spain reported a 50.5 % prevalence (Mainar et al. 1998).

Also, chlamydiosis is considered the main cause of lamb loss in Europe (Livingstone et al. 2009).

The most widely used diagnostic method for detection of *C. abortus* infection in animals is the enzyme-linked immunosorbent assay (ELISA). However, polymerase chain reaction (PCR) as a new diagnostic tool has proven to be sensitive and reliable (Berri et al. 2009; Navarro et al. 2009; Rekiki et al. 2002; Sachse et al. 2009a, b) and has become an important diagnostic method for *C. abortus* infection in animals. This technique has been widely used for detection of *C. abortus*: in caprine abortions in Italy, this pathogen was detected in 0.6 % of the studied population; samples were taken from muscle, liver, abomasum, spleen and placenta tissue. A follow-up study showed that *C. abortus* was the second most common pathogen in placenta tissue from aborted kids (Masala et al. 2005, 2007). *C. abortus* was also detected via PCR in buffalo and sheep semen, and in the same study, it was determined that the detection threshold was 0.25 inclusion forming units (IFU) for PCR and 4.0 IFU for cell culture isolation (Amin 2003). Marsilio et al. (2005) showed that PCR performed on 117 sheep vaginal swab samples exhibited high specificity and sensitivity for *C. abortus* diagnosis in animals showing chlamydial infection symptoms. Moreover, they proved that swab sampling allowed safer manipulation of infectious material. The best diagnostic results can be obtained when more than one method is used. A study performed in Tunisia using 50 serum samples and vaginal swabs of adult ewes showed the presence of *C. abortus* in 58 and 18 % of the individuals sampled using ELISA and cell culture, respectively (Rekiki et al. 2002). These results were confirmed by PCR-restriction fragment length polymorphism (RFLP) analysis of sequences found in the 16S-23S ribosomal DNA (rDNA) intergenic spacer (Gutierrez et al. 2012; Rekiki et al. 2002).

Different primers have been designed to detect *Chlamydophila spp.* using PCR. Greco and collaborators tested four sets of primers (U23F/23Sigr, 16SF2/23R, CTU/CTl and CpsiA/CpsiB) to detect *C. abortus* in tissue samples from aborted foetuses (Greco et al. 2005). In a previous study, we reported a primer pair (CpXal-1/CpXal-2) and successfully detected *C. abortus* in sheep (Jiménez-Estrada et al. 2008).

In Mexico, *C. abortus* infections have been underdiagnosed in humans and other animals; also, there are just a few published studies. In Mexico, this pathogen was first detected in 1996 in adult sheep faecal samples using cell culture (Escalante-Ochoa et al. 1997). Subsequent publications reported isolation in aborted goats, and in 2008, *C. abortus* was detected in 0.65 % of ewes with a history of abortion and also in lung and liver tissue samples from aborted foetuses (Jiménez-Estrada et al. 2008). In Mexico, there is a significant population of goats; however, *C. abortus* infection prevalence has not yet been reported for this animals. Therefore the aims of the present study are to determine seroprevalence in goat flocks and to demonstrate the circulation of *C. abortus* in dairy goat farms in the municipality of Guanajuato, Mexico, using PCR and cell culture.

Materials and methods

Ethics statement

Animal conditions complied with the recommendations of the Council for International Organizations of Medical Science (WHO 1985) and NOM-062-Z00-1999 (NOM 1999).

This study was approved by the bioethics' Institutional Committee of the Centro de Investigación y Estudios Avanzados en Salud Animal at UAEMex, Toluca, Mexico, with protocol number 2230/2006U. Since the initial serological diagnosis of clinical abortion was attributable to zoonotic bacterial diseases, intensive efforts were made to maximize the owners' participation. Verbal consent from owners/managers was obtained.

Study sites

The study was carried out in nine commercial dairy goat farms in the state of Guanajuato, which is located in the central region of Mexico, between 19° 55′ 08″ and 21° 52′ 09″ North latitude and between 99° 36′ 06″ and 102° 05′ 07″ West longitude. Dairy farms had a total population of 2,582 Saanen goats, and the flocks ranged from 97 to 318 animals. All farms included in the study have mechanical milking facilities and milk refrigeration tanks. Sanitary, economic and production data were available. Sanitary records stated that flocks were free of brucellosis and that clinical and subclinical mastitis rates were 0.3 and 23 %, respectively. Flocks were intensively managed. Forage was a mixture of alfalfa, maize silage and a commercial concentrated mixture with 18 % protein. Breeding was carried out through natural mating or artificial insemination, with an average of one kid born per doe per year. Sire bucks from the USA were introduced in 2003, and subsequent replacements came from the same place.

Sample size for stratified random sampling

Dams (n=246) were selected randomly from nine farms with a total goat population of 2,582. Predicted *C. abortus* prevalence was 20 %, and this was based on the number of females with reproductive problems within the flocks. Sample size was calculated through a method to estimate prevalence in a large population, with a confidence level of 95 % and an absolute precision of 5 % (Thrusfield 2007). Age and previous abortions data were recorded for each sampled animal.

Serological testing

Serology Blood samples were collected by jugular venipuncture using a Vacutainer system. Total blood samples were centrifuged at 1,728×*g* for 10 min. Sera were collected and kept at −20 °C. Recombinant ELISA (rELISA) was performed according to the manufacturer's protocol.

Isolation and molecular identification Sterile cotton-tipped plastic swabs were used for vaginal and rectal sampling. Foetal lung and spleen tissue samples from a sudden abortion were also collected. Vaginal and rectal swabs and foetal tissue samples were kept in sucrose-phosphate-glutamine buffer (SPG) supplemented with 10 % foetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 50 μg/ml gentamicin). Samples were transported at 4 °C and kept at −20 °C until further use (Escalante-Ochoa et al. 1997). Vaginal swabs were squeezed out against the wall of the tube and centrifuged at 1,728×*g* for 30 min. The supernatant was then transferred to 1.5-ml microcentrifuge tubes, SPG buffer was then added and samples were stored at −80 °C. Rectal swab samples were processed the same way. Organ samples from the aborted foetus were homogenized in SPG and centrifuged at 1,728×*g* for 20 min at 4 °C, and SPG was added (1:1 *v*/*v*). Samples were washed twice by centrifugation, and the supernatant was transferred to 1.5-ml microcentrifuge tubes and kept at −80 °C until used (Creelan et al. 2000).

ELISA test

Sera were tested for the presence of IgG antibodies against *C. abortus* using rELISA (version P00700/04-18/02/05, Institut Pourquier, Montpellier, France). The microtitre plates (96 wells) were supplied coated with the recombinant protein. Sera were diluted 1/20 in assay diluent in the plate wells and incubated for 1 h at 37 °C and then washed with washing solution. The conjugate, a peroxidase-linked anti-ovine IgG polyclonal antibody, was added to the wells and incubated at the same temperature. The plates were then washed. Enzyme substrate and chromogen tetramethylbenzidine (TMB) were added and incubated for 20 min at 21 °C. Enzymatic reaction was stopped by acidification, and the resulting optical density (OD) was measured at 450 nm using a microtitre plate reader (Digiscan, Asys Hitech, Eugendorf, Austria). Positive and negative control sera were supplied by the manufacturer. The final values were expressed as sample/positive control percentage (S/P %). Sera with S/P % equal to or lower than 50 % were considered negative, sera with an S/P % between 50 and 60 % were doubtful and sera with an S/P % >60 % were considered to be positive. This rELISA assay uses a recombinant fragment of an 80–90-kDa *C. abortus* protein. The test is highly specific; it does not react with sera from SPF-lambs who were experimentally infected with various subtypes of *Chlamydophila pecorum*. It also does not produce any false-positive results with reference sera known to be free from ovine ezootic abortion (OEA), nor with sera from flocks with a suspected *C. pecorum* infection or with no clinical history of abortions. rELISA proved to be more sensitive than the CFT and other ELISA tests, using sera from pregnant ewes experimentally infected with *C. abortus*. The test detected more positive sera from infected ewes lambing normally than any other assay. However, in flocks where the spread of the OEA was limited, rELISA was less satisfactory and was unable to differentiate between naturally infected and vaccinated animals (Vretou et al. 2007; Wilson et al. 2009).

Cell culture isolation

HeLa 229 cells were cultured in Eagle's minimal essential medium (MEM), supplemented with 10 % FBS, 2 mM glutamine, non-essential amino acids, 2 mM gentamicin and 0.02 % vancomycin (Siarkou et al. 2002). Cells were incubated at 30 °C under 5 % CO_2. Cell monolayers were prepared in 96-well polystyrene plaques by adding 1.2×10^4 cells/150 μl/well. When confluence reached 70–80 %, MEM was removed. Cells were washed twice for 1 min with 100 μl PBS (pH 7.4) per well. Frozen stored samples were thawed and added (20 μl per well) to HeLa cell cultures. Microtitre plates were incubated for 1 h at 37 °C in a saturated humidity environment and 5 % CO_2. After incubation, 130 μl of supplemented MEM was added, and plaques were again incubated as previously mentioned for 72 h. This isolation procedure was performed twice, with and without a coverslip on microtitre plaques during the last 72 incubation hours. Microtitre plates incubated with a coverslip were evaluated using direct immunofluorescence to detect cytoplasmic inclusions of *Chlamydophila* spp. Microtitre plates incubated without a coverslip were kept as backup in case isolation from covered samples was not successful. Inoculated cell cultures were subpassaged three times.

PCR

DNA from vaginal, rectal and aborted tissue samples was extracted by alkaline lysis and standardized at 100 ng/μl. PCR was prepared with 1 μl purified DNA, 0.2 μM of forward and reverse primers, 200 mM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP and dTTP), 1.5 mM $MgCl_2$ and 0.25 U Taq DNA polymerase and endonuclease-free double-distilled water for a 25-μl total volume reaction. PCR was performed in an automatic thermal cycler under the following conditions: preincubation at 94 °C for 2 min, then 30 cycles of 94 °C for 40 s, 60 °C for 2 min, 72 °C for 2 min and a final incubation of 72 °C for 10 min.

The *C. abortus* AB7 strain was included as a positive control and distilled water as a negative control. Amplification of the polymorphic outer membrane protein (POMP 90-91B) gene was carried out with primers CpXal-1 (5′-ACGGTCACTT GGAAACAAGG-3′) and CpXal-2 (5′-AGCAGAGGTTGG GCTCACTA-3′), which hybridize in the region of 1,541 to 2,452 bp of the POMP 90-91-B gene and amplified a 912-bp product (Jiménez-Estrada et al. 2008). DNA amplicons were visualized through electrophoresis in 2.5 % agarose gels with 4 μg/μl ethidium bromide.

Results

C. abortus seroprevalence determined by rELISA in Saanen breed does from farms sampled in Guanajuato, Mexico, was 4.87 % (n=246 goats). Positive goats were found in 66.6 % (six of nine) of sampled flocks, and prevalence among farms ranged from 3.44 to 13.51 %. It was not possible to isolate *C. abortus* using tissue culture or to detect it by PCR from either vaginal or rectal swabs, or serum samples from adult seropositive goats. It was not possible either to isolate bacteria from foetal tissue samples. However, we succeeded in detecting the bacteria through PCR from DNA extracted from spleen foetal samples (Fig. 1).

Fig. 1 Agarose gel electrophoresis of *Chlamydia abortus* POMP 90-91B gene PCR amplicon. *Lanes 1 and 6*, molecular weight marker 1 kb; *2*, *C. abortus* AB7* (positive control, 912-bp band); *3*, negative control; *4*, clinical sample of the abortion (912-bp band); *5*, empty lane. *Strain donated by the Departamento de Salud Animal, Facultad de Medicina Veterinaria, Universidad de Murcia, Spain

Discussion

In this study, serologic diagnosis was based on a rELISA kit that has been previously demonstrated to be more specific and sensitive than CFT and other ELISA tests. It does not present cross-reactivity with *C. pecorum* or enterobacteria. It does not produce any false-positive results with reference sera known to be free from OEA or with sera from flocks with no clinical history of abortions. This rELISA test kit has been reported to detect more positive sera from infected ewes lambing normally than any other assay (Vretou et al. 2007; Wilson et al. 2009). In this study, through rELISA, we demonstrated that *C. abortus* is circulating in goat dairy farms in the state of Guanajuato, México. Here, we report a seroprevalence of 4.9 % (12/246). Previously, our laboratory reported 22.6 % of prevalence in an open population of ewes from the state of Mexico (Jiménez-Estrada et al. 2008). Considering that both states (Mexico and Guanajuato) are located in central Mexico, we were expecting to find a similar seroprevalence anti-*C. abortus* between the two animal populations (goats and sheep). Prevalence was lower in goats compared to that in sheep, and it could be hypothesized that *C. abortus* has a larger prevalence in the state of Mexico than in the state of Guanajuato; further studies should be conducted to confirm this hypothesis. However, this difference could also be explained by the differences in flock management, since animal production systems are quite different: while the sheep of the state of Mexico are kept under a traditional extensive husbandry system, the goats included in the present study are maintained under an intensive and modern husbandry system. Herds included in the present study separate kids at birth to be fed with pasteurized colostrum and milk. Further studies need to be conducted to find out if prevalence remains the same in animals kept under a more rustic husbandry system in Guanajuato. However, even if prevalence is not as high as expected, these results are relevant since *C. abortus* is a pathogen that affects the production efficiency of dairy goat farms and it may have an impact in reducing milk and kid yields per year. On the other hand, *C. abortus* is a zoonotic pathogen that represents a public health risk that may also cause abortion in infected women (Rodolakis and Yousef 2010), and therefore, it is important to detect infected goat flocks and establish actions to prevent human infection.

Considering that rELISA results in flocks with limited spread of the OEA were less satisfactory, we decided to use PCR as a support diagnostic test. Molecular identification of *C. abortus* in foetal samples found in the present study confirmed previous findings about the presence of this bacteria in ruminants in Mexico (Escalante-Ochoa et al. 1997; Jiménez-Estrada et al. 2008). Detection of *C. abortus* with PCR from placental or foetal tissue has been previously reported by others (Masala et al. 2005, 2007). Our findings support the

idea that the PCR diagnostic test is a useful tool to detect *C. abortus* from placental or foetal tissue samples (Masala et al. 2005, 2007). We had limited success in our attempt to detect the pathogen with PCR from vaginal or faecal swabs. These findings were unexpected because it has been previously reported that the PCR diagnostic test from these kinds of samples is very sensitive (Livingstone et al. 2009; Marsilio et al. 2005). There are some variables that may reduce the amount of bacteria to undetectable numbers, which makes PCR-based detection and bacteria isolation more difficult. For example, isolation can be achieved at a higher level of success when animals are sampled at the beginning or at the end of pregnancy than when sampling is carried out at midgestation (Michalopoulou et al. 2007). It has also been shown that the chlamydiae are normally shed more abundantly in estrus than during the diestrus period (Livingstone et al. 2009). In the present study, does were sampled in October and November when most animals are at midgestation stage, which might explain the low detection efficiency. This should be taken into consideration in future studies. There are other problems associated to *C. abortus* diagnosis that should also be considered when choosing diagnostic methods. For example, PCR-mediated detection of *C. abortus* does not always correlate with successful isolation and culture of the bacteria from tissue samples. It has been reported that *C. abortus* was more frequently detected by PCR in healthy seropositive goats than in goats with a history of abortion (Wang et al. 2001). We limited our study to the search for *C. abortus*, and therefore, PCR primers used were chosen to specifically detect this species and were unable to detect *C. pecorum*, another chlamydial species of ruminants that could potentially induce abortion in small ruminants. This pathogen has not been reported in Mexico. However, it would be interesting to search for this pathogen in future studies.

Conclusions

The results from rELISA and PCR showed that *C. abortus* is present in dairy goat farms in the state of Guanajuato. Considering the implications of this disease in public health and the economy, it is necessary to carry out further research on prevalence and distribution of *C. abortus* to better understand its impact in animal health and production in Mexico and to alert health authorities so that they can implement pertinent measures to control and prevent *C. abortus* transmission among animals and humans (Jimenez-Estrada et al. 2008).

Conflict of interests The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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