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Campylobacter fetus subsp. *fetus* ovine abortion outbreak in Argentina - Brote de abortos ovinos por Campylobacter fetus fetus en Argentina

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Abstract

Campylobacter infection is one of the major causes of ovine abortions worldwide. However there are no previous reports of *Campylobacter fetus* subspecies *fetus* associated to outbreak ovine abortion in Argentina. This study constitutes the first report of *C. fetus fetus* outbreak ovine abortion in naturally infected ewes in Argentina. The problem was presented in a dairy flock of 205 pampinta ewes. In one week 7 abortions were recorded. Some of the aborted sheep also retained placentas and showed vaginal discharges. Twins and three placentas were sampled to determine the cause of the abortions. *C. fetus fetus* was isolated in lung, liver and abomasal fluids from both fetuses and in 2 out of 3 placentas. C. fetus fetus DNA was detected in 2/3 placentas tested and in lungs of both fetuses. Additionally, N. caninum DNA was amplified in a ewe placenta. No N. caninum or T. gondii serological tests were carried out in the ewes, therefore these protozoa infection could not be ruled out in the flock. Toxoplasma and Neospora infection are relatively common in Argentinean flocks. However, protozoan abortion in small ruminants has not been extensively reported, therefore it is important to rule out the presence of these pathogens when ovine abortions are registered. Further work is needed in order to quantify the impact of *C. fetus fetus* infection in sheep production systems of the region.

Keywords: *Campylobacter fetus* subsp. *fetus*, ewe, abortion, Argentina

Resumen

El aborto por *Campylobacter* es una de las causas más importantes de abortos en ovinos a nivel mundial. El presente constituye el primer reporte de *C. fetus fetus* asociado a un brote de abortos en ovinos infectados naturalmente en Argentina. El problema se presentó en un rebaño lechero de 205 ovejas pampinta. En el transcurso de una semana se observaron un total de 7 abortos, algunas de las ovejas abortadas presentaron además retención de placenta y descargas vaginales. Se tomaron muestras de coderos mellizos abortados y de 3 placentas de ovejas abortadas. *C. fetus fetus* fue aislado a partir de pulmón, hígado y líquido de abomaso de ambos fetos y de 2 de las 3 placentas colectadas. ADN de *C. fetus fetus* fue detectado en 2/3 placentas y en los pulmones de ambos fetos. Además AND de *N. caninum* fue amplificado en una de las placentas, sin embargo la infección por dicho protozoo no pudo ser confirmada ya que no se realizaron test serológicos para *N. caninum* ni para *T. gondii.* Si bien las infecciones por *Toxoplasma* y *Neospora* son relativamente comunes en Argentina, el aborto por protozoos en pequeños rumiantes no ha sido ampliamente reportado, por lo cual es importante descartar la presencia de estos patógenos cuando se registran abortos ovinos. Futuros trabajos serán necesarios para cuantificar el impacto de la infección por *C. fetus fetus* en los sistemas de producción ovina de la región.

Introduction

Ovine abortion is particularly relevant in the economy of countries where lamb is the predominant source of animal protein [1,2]. Infectious ovine abortions have been related with different bacterial, viral and protozoal infections. Among bacteria, *Campylobacter* spp., *Listeria monocytogenes*, *Brucella* spp., *Salmonella* spp., and *Chlamydia abortus* are the most commonly associated reproductive wastage [3].

Campylobacter species are microaerophilic Gram-negative bacteria and they are recognized veterinary and human pathogen [4,5]. *C. fetus* comprises two subspecies, with two distinct diseases of veterinary importance: *C. fetus* subsp. *venerealis* causes enzootic infertility and abortion in cattle, while *C. fetus fetus* is associated with epizootic abortion in cattle and sheep [4,6]. In the United Kingdom, it was diagnosed 18% of ovine abortions in 1999 related to *Campylobacter* spp. [7]. *C. fetus* is the most frequently species associated with ovine abortions in the United Kingdom, Australia and New Zealand, compared with the United States where *C. jejuni* is the most prevalent species [8-11].

Healthy sheep can be asymptomatic carriers of *Campylobacter* species in their intestine and gallbladder while some other strains can cause systemic infections [12]. In susceptible pregnant ewes, initial bacteremia is followed with placentitis, fetal infection, and abortion, which usually occurs in the last trimester of pregnancy. During the initial period of infection, ewes usually do not show clinical signs of disease; however, occasionally ewes succumb due to uterine sepsis and septicemia if fetus is retained in utero [6].

Besides the veterinary importance of *C. fetus fetus* infections, this pathogen is associated with a variety of infections in humans including: bacteraemia, gastrointestinal disease, meningitis in infants and adults, cellulitis, cardiovascular disease and abortion [13-19].

Preventive and control management of abortions are pathogen-specific. Therefore, accurate and rapid etiological diagnostic is necessary. To our knowledge, this work reports for the first time the identification of *C. fetus fetus* as the cause of abortion in dairy ewes in Argentina.

Materials y methods

Flock description

A 205-Pampinta dairy ewe flock was allocated at the Instituto Nacional de Tecnología Agropecuaria (INTA) in Anguil, La Pampa province, Argentina (36º 32' 31" S, 63º 59' 28" W). Ewes were grazing alfalfa pastures under semi-intensive conditions and no records of similar outbreaks were registered.

In February 2014, 7 ewes aborted full-term fetuses during a week. Some of the aborted ewes also retained placentas and showed vaginal discharges. Twin lambs (aborted ewe #1) and three placentas (aborted ewes #2, #3 and #4) were submitted for to the Veterinary Diagnostic Laboratory at INTA in Balcarce, Buenos Aires province, Argentina.

Necropsy and sampling

Post mortem examination was conducted and samples of placentas and tissues from aborted fetus were collected. Tissue samples from aborted fetus including brain, myocardium, lung, liver, kidney and placenta were fixed in 10% neutral buffered formalin, and processed routinely for the production of 4-µm-thick sections and stained with hematoxylin and eosin for histological examination. Placentas, abomasal fluid, lung and liver from aborted fetus were processed routinely for bacterial cultures. Thoracic and abdominal fluids from the aborted fetus were processed for the detection of antibodies against *Toxoplasma gondii* and *Neospora caninum* by indirect fluorescent antibody test (IFAT). Frozen heart, brain, liver, lung and placental tissue samples were processed for the detection of *T. gondii*, *N. caninum* and *Chlamydaceae* DNA by polymerase chain reaction (PCR).

Bacteriological studies

All tissues were cultured on Columbia Blood Agar (CBA) (Oxoid Ltd., Wad Road, Basingstoke, UK) with 7% sterile defibrinated bovine blood and Skirrow agar (SK) with antibiotics [20]. CBA and SK plates were incubated at 37ºC under 10% CO_2 and microaerobic atmospheres (5% O_2, 10% CO_2 and 85% N_2) respectively and examined daily during 7 days. All isolates were identified by routine procedures. Isolated *C. fetus* was biochemical tested for typification accordingly [21].

DNA extraction

Genomic DNA was extracted from pure cultures of *C. fetus fetus* using methods based on standard procedures. Briefly, single colonies were resuspended in 50 µL sterile ultrapure water and incubated in a 100°C heating block for 10 min. Lysates were centrifuged and the cell-free supernatant transferred to a fresh tube. DNA extracts were stored at -20°C.

Tissue samples DNA was extracted from placentas and fetal tissues with a commercial kit (DNeasy, Blood and Tissue kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions and stored at -20ºC.

Campylobacter speciation by PCR

Campylobacter fetus fetus DNA was amplify using a multiplex PCR assay described previously by Iraola et al. [22], with modifications: 2 µL of DNA was used in a 25 µL reaction containing 0.2 mM of each dNTP (Promega, USA), 2 µL of 1X reaction buffer (Promega, USA), 2 mM MgCl2 (Biodynamics, Argentina), 0.625 µM MG3F/MG4R primer set, 0.375 µM nC1165g4F/nC1165g4R primer set, and 1.25 U Taq DNA polymerase (Promega, USA). A PCR Thermal Cycler (Thermo Fisher Scientific, USA) was used for amplification. The following cycling conditions were used: an initial denaturation for 3 min at 95ºC followed by 35 cycles of denaturation for 30 sec at 94ºC, annealing for 30 sec at 53ºC, extension for 1 min at 72ºC and a final extension for 10 min at 72ºC. Multiplex PCR use these two primer sets: MG3F/MG4F amplifies a 764-bp region for *C. fetus fetus*, and the set C1165g4F/nC1165g4R amplifies a 233-bp region that is only present in *C. fetus venerealis*. PCR products were separated in 1.0% agarose gel and stained with SyberSafe (Invitrogen, USA)

T. gondii and *N. caninum* PCR

Toxoplasma gondii PCR was carried out using the primer pairs Tox5/Tox-8 described by Herrmann *et al*. [23]. *N. caninum* semi-nested PCR was carried out using the primer pairs NcNN1/NcNN2 and NcNP1/NcNP2 [24,25]. Specific amplification products of 451bp and 249bp were considered positives for *T. gondii* and *N. caninum*, respectively.

Chlamydiaceae Real Time PCR

A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene was used in this study [26]. The protocol includes primers Ch23S-F (*5'-CTGAAACCAGTAGCTTATAAGCGGT-3'*), Ch23S-R (*5'-ACCTCGCCGTTTAACTTAACTCC-3'*), and probe Ch23S-p (FAM-5'-CTCATCATGCAAAAGGCACGCCG-3'-TAMRA). Each reaction mix container 5 µL sample DNA template, 12.5 µL of Universal Master mix 2X (Applied Biosystems), 1.5 µL of each primer (300 nM) and 1 µL of the probe (200 nM), and 3.5 µl deionized water. The temperature-time profile was as follows 95°C 10 min, 50 cycles of 95°C 15 s, 60°C 60 s.

T. gondii and *N. caninum* indirect fluorescent antibody test

Antibodies against *T. gondii* and *N. caninum* were identified in fetal fluids by IFAT according to the technique described by Hecker et al. [27]. A test was considered positive if the titer was ≥ 1:10 [28,29].

Results and discussion

Campylobacter fetus fetus ovine abortions frequently occurred in the last third of gestation [6]. According to the records of this affected flock, fetuses were at week 10-13 of gestation at the moment of abortion. At necropsy, both fetuses were autolytic and with initial stages of mummification (Fig. 1): serosanguineous fluids were observed in their thoracic and abdominal cavities although the surfaces of internal organs were homogenously pink-tinted and dry. Brains were autolytic. Other tissue lesions has been macroscopically described in *C. fetus* aborted fetuses characterized by circular white liver foci [10]. Nevertheless, autolytic changes observed in these fetuses could be masked any other gross lesion. Ewe #2 and #3 showed autolytic placentas whereas ewe #4 placenta was minimally autolytic and intercotyledonary areas were edematous and hyperemic. Similar placental lesions have been described in *Campylobacter* ovine and bovine abortions [30,31].

Microscopic lesions observed in fetuses and placentas were consistent with those previously reported by other authors [30]. Suppurative bronchopneumonia with an accumulation of neutrophils in the bronchi and bronchiole, and suppurative pericarditis were present in both fetuses. Other tissues showed different degrees of autolysis which could probably be masked any other pathological changes.

Despite the high degree of autolysis observed in fetal and placental tissues, *C. fetus fetus* was isolated in lung, liver and abomasal fluids from both fetuses and in 2 out of 3 placentas (ewes #3 and #4). Several authors have highlighted problems associated with the isolation of the organism due to its reduced viability under normal atmospheric conditions and rapid overgrowth of more vigorously multiplying contaminating organisms [32]. It is strongly recommended the use of a selective growth medium as SK agar, particularly

when placentas are cultured where other contaminating bacteria usually develop.

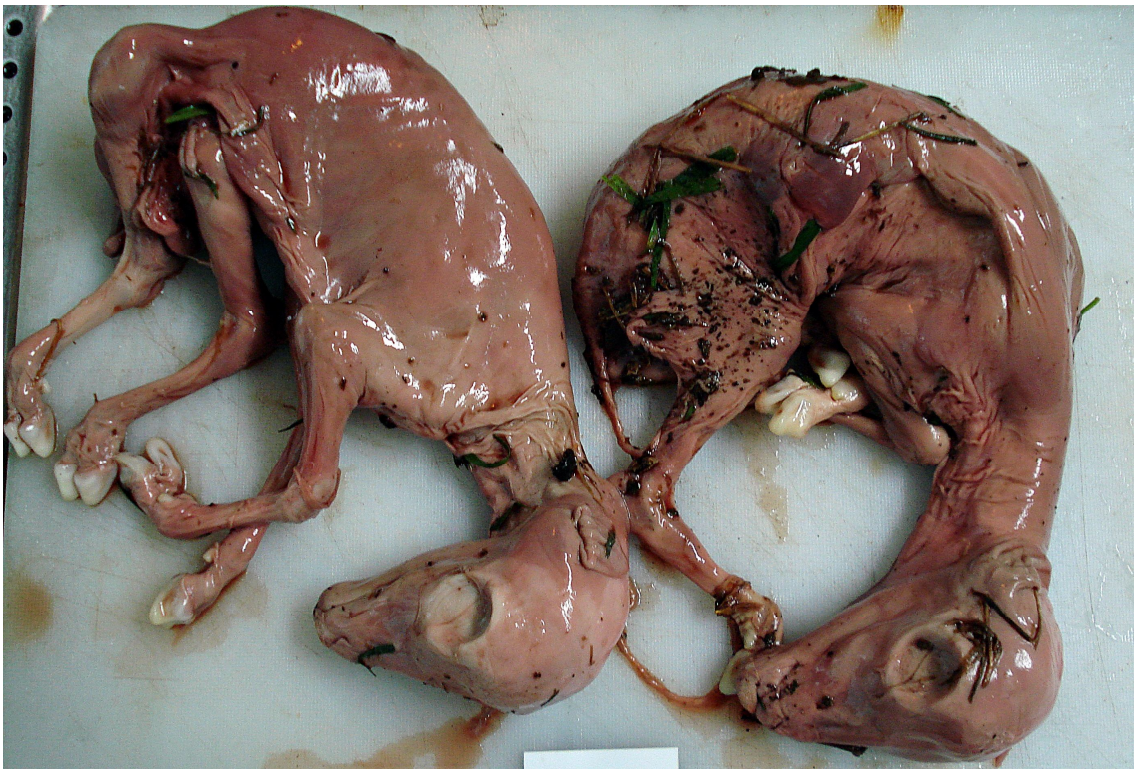

Fig. 1: Aborted ovine fetuses from which *Campylobacter fetus fetus* was the only agent isolated. It is noted mummification and autolysis.

C. fetus fetus DNA was detected in 2/3 placentas tested (ewes #3 and #4) and in lungs of both fetuses (Fig. 2). Rapid identification of the causative agent by the use of molecular techniques in comparison to traditional bacteriological methods demonstrate the importance of PCR techniques as a practical alternative to laboratory diagnosis. This is particularly important in the diagnosis of fastidious microorganisms such as *Campylobacter* genus [33].

No *T. gondii* and *Chlamydaceae* infection were detected in fetal and placental tissues analyzed using IFAT and PCR. Interestingly, *N. caninum* DNA was amplified in ewe #2 placenta. No *N. caninum* or *T. gondii* serological tests were carried out in the ewes, therefore these protozoa infection could not be ruled out in the flock. However, no lesions usually associated with protozoan abortions were observed in fetal tissues. *Toxoplasma* and *Neospora* infection are relatively common in Argentinean flocks [27]. However, protozoan abortion in small ruminants has not been extensively reported [34], therefore it is important to rule out the presence of these pathogens when ovine abortions are registered.

In New Zealand, the application of an inactivated vaccine of *C. fetus fetus* (strain A5915; Campylovexin, Schering-Plough Although Animal Health Ltd, New Zealand) is used for the prevention of *Campylobacter* ovine abortions [35]. Although *Campylobacter* vaccination has been used worldwide for controlling reproductive losses in sheep, this pathogen remains a major cause

of sheep abortions [8]. Large genetic and antigenic variation of *Campylobacter* strains and insufficient cross-protection between the immunogen and wild-type bacteria could probably contribute to vaccine failure [8]. Aborted sheep usually clean *Campylobacter* infection a few weeks after abortion and long-lived immunity is established following abortion and they do not usually remain as carriers and conceive in the future reproductive season [36].

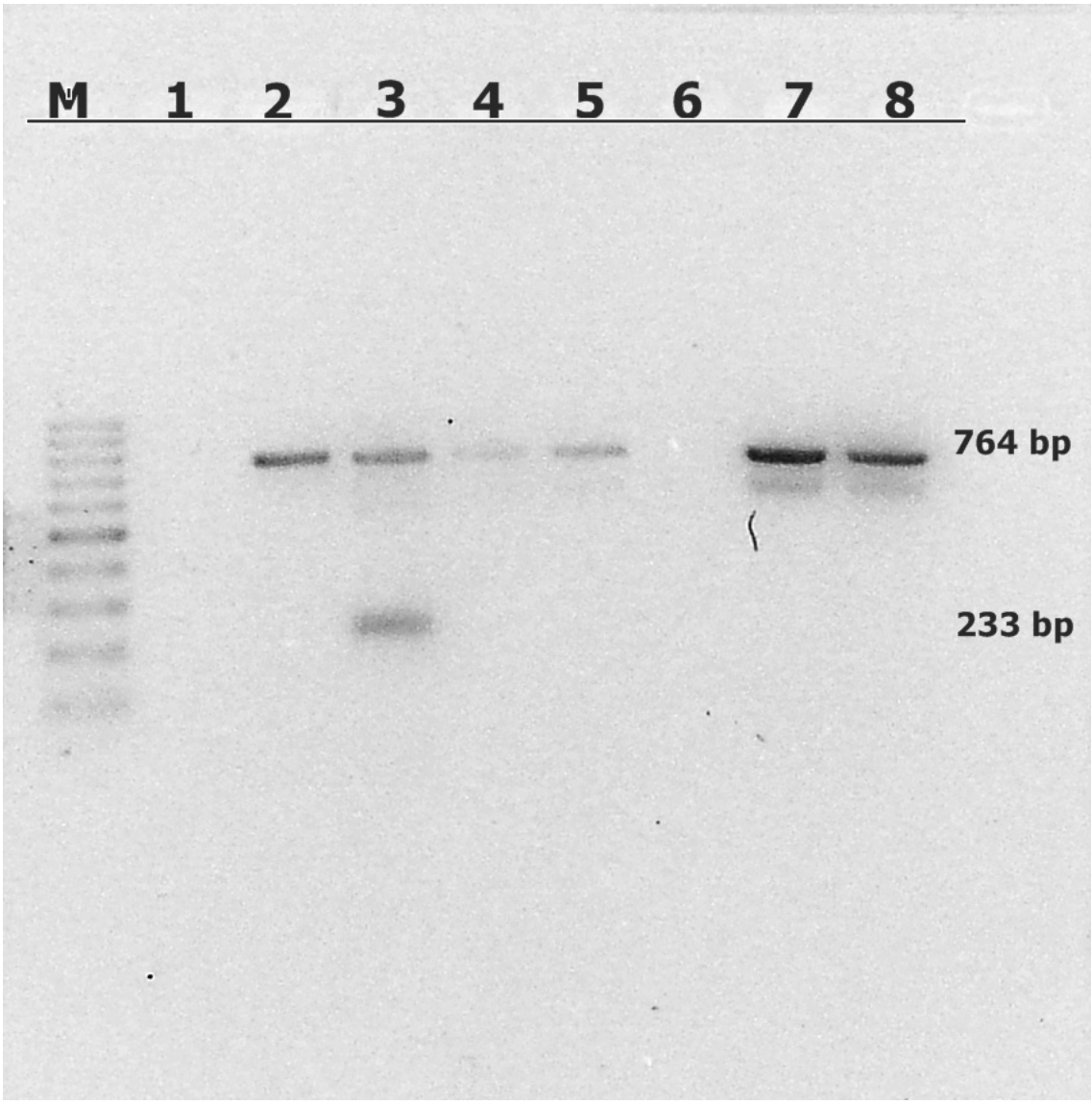

Fig. 2: *Campylobacter* speciation by PCR**.** Multiplex PCR use two primer sets: MG3F/MG4F amplifies a 764-bp region for *C. fetus fetus*, and the set C1165g4F/nC1165g4R amplifies a 233-bp region that is only present in *C. fetus venerealis*. Lanes M: Molecular marker 100 pb; lane 1: negative control; lane 2 control *Campylobacter fetus fetus*; lane 3 control *Campylobacter fetus venerealis*; lanes 4-5 fetal lungs; lane 6: placenta ewe #2; lane 7: placenta ewe #3; lane 8: placenta ewe #3.

In addition to vaccination, some authors emphasized the importance of other management techniques to avoid infection: reducing levels of exposure to the infected material, avoiding contaminated pastures, reducing stocking density,

removing aborted material, segregating aborted ewes from susceptible young sheep and avoiding mechanical transmission, among other factors. These tools are important for the prevention of the spread of this disease [35]. Until the present, Argentinean flocks are not routinely vaccinated against *Campylobacter* infections and the currently available vaccines are designed to reduce bovine genital campylobacteriosis. Future works are needed in order to evaluate the inclusion of an immunogen in local flocks.

As previously was mentioned, the importance of *Campylobacter* abortion occurrence is given not only by the loss in production but also by public health risks [37]. This paper describes the first report of *C. fetus fetus* ovine abortion in Argentina. Further works are needed in order to quantify the impact of this infection in the sheep production systems of the region.

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