First isolation and characterization of Chlamydophila abortus *from abortion tissues of sheep in Sardinia, Italy*

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Veterinaria Italiana 2013, **49** (4), 331-334. doi: 10.12834/VetIt.1303.10
Accepted: 28.10.2013 | Available on line: 18.12.2013

Keywords
Cell culture isolation, *Chlamydophila abortus*, Ovine abortion, PCR.

Summary
Chlamydophila abortus (*C. abortus*) is the responsible agent for chlamydial abortion [commonly known as Enzootic Abortion of Ewes (EAE)] and, as such, it causes major financial losses to the sheep industry worldwide. Isolation of the pathogen is considered the 'gold standard' and most sensitive method of detection for diagnosing chlamydial infection. So far, there has been no isolation of *C. abortus* from ovines in Sardinia, Italy. This letter describes the results of a study conducted on a total of 89 aborted samples (40 foetuses and 49 placentae) collected in 2010 in Northern Sardinia, Italy. Three placentae resulted PCR-positive when analyzed using the putative outer membrane protein (pmp) specific primers, the test lead to the identification and first isolation in cell culture of *C. abortus*. This letter to the editor describes the first isolation of *C. abortus* from ovine placentae and increases the knowledge of one of the agents that causes ovine abortion in Sardinia and, more generally, in the Mediterranean basin.

Primo isolamento e caratterizzazione di Chlamydophila abortus *da aborti ovini in Sardegna, Italia*

Parole chiave
Aborti ovini, *Chlamydophila abortus*, Isolamento in colture cellulari, PCR.

Riassunto
Chlamydophila abortus (*C. abortus*) è l'agente responsabile dell'aborto da chlamydia [comunemente conosciuto come Enzootic Abortion of Ewes (EAE)] e provoca gravi perdite economiche per il comparto ovino in tutto il mondo. L'isolamento del patogeno è considerato il "gold standard" e il metodo più sensibile di rilevamento per la diagnosi di infezione da *Chlamydia*. Nessun dato è attualmente disponibile riguardo l'isolamento di *C. abortus* da ovini in Sardegna, Italia. Lo scopo di questo studio è stato quello di determinare la presenza di *C. abortus* durante la stagione degli aborti ovini nel 2010. Un totale di 89 campioni d'aborto (40 feti e 49 placente) provenienti dal Nord della Sardegna sono stati analizzati; 3 placente sono risultate positive alla ricerca del DNA di *C. abortus* in PCR utilizzando primers specifici per 'putative outer membrane protein' (pmp), portando all'identificazione ed al successivo isolamento in coltura cellulare di *C. abortus*. Questo lavoro documenta il primo isolamento di *C. abortus* da placenta ovina e integra la conoscenza riguardo uno degli agenti abortigeni circolanti in Sardegna e, più in generale, nel bacino del Mediterraneo.

Chlamydophila abortus is a zoonotic Gram negative bacterium, it infects epithelial cells and monocyte/macrophages of a wide host range, resulting in a broad spectrum of diseases in humans, as well as in other mammals and birds (3). *C. abortus* is also one of the most common causes of infectious abortion in sheep and goats worldwide, the so called enzootic abortion of ewes (EAE). The disease has a major economic impact as it represents the most important cause of lamb loss in sheep in several parts of Europe, North America and Africa (6).

In Sardinia, the second largest island of Italy in the middle of the Mediterranean Sea, sheep farming is a very important sector of primary animal production both economically and socially. In this reagion, the primary sector is still of outstanding importance, especially sheep rearing; in the island there are 32,209 farms, of which 14,854 are sheep farms with a total of 3,140,442 sheep corresponding to nearly half of the total Italian sheep population. These farms are registered in the Italian National database (BDN data 2012, Reg. CE 1760/2000).

The incidence of ovine abortion is very high because of the high animal density (149/km^2 against 23/km^2 on the mainland), poor breeding conditions (traditional herding practices and transhumance) and inadequate prophylaxis. The economic losses due to lamb mortality and reduced milk production are estimated at about 10 million euro per year (6).

Chlamydial studies based on clinical and serological features were conducted in Sardinia using indirect immunofluorescence antibody tests to detect antibodies against *C. abortus*. Moreover, PCR assay was then used to identify *C. abortus* in samples from adult ovine abortions (6). Historically, the isolation of the pathogen has been considered the 'gold standard' and most sensitive method of detection for diagnosing chlamydial infection. Isolation involves the cultivation of organisms from clinical samples in either embryonated hen's eggs or cell culture. So far, there has been no direct detection or isolation of chlamydiae in Sardinia. The aim of this study was to detect and isolate *C. abortus* from ovine placental tissues in Sardinia.

A total of 89 ovine aborted samples (40 foetuses and 49 placentae) were brought to the Istituto Zooprofilattico Sperimentale della Sardegna. The aborted-sheep came from semi-wild flocks and it was not possible to distinguish primiparae from pluriparae sheep. The 49 placentae and the 40 foetuses (40 brains, 3 livers and 6 spleens) were then analyzed for detection and identification of *C. abortus* antigens using acetone-fixed tissue sample smears. Slides were subsequently analyzed by a direct immunofluorescence (IFD) test (IMAGEN *Chlamydia* test, Oxoid, Ely, United Kingdom) containing fluorescein isothiocyanate (FITC) conjugated monoclonal antibody that detect elementary bodies of *C. abortus*.

One hundred microliters of genomic DNA from 1000 µl of total homogenated organs were extracted using QIAgen columns (QIAamp tissue kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Chlamydial DNA was amplified by using oligonucleotide primers PMP-1 [5'-ATGAAACATTCCAGTCTACTGG-3'] and PMP-2 [3'-TTGTGTAGTAATATTATCAAA-5'] targeted to the outer membrane protein (POMP 90-91B) gene which amplify a product of 320 bp (2). A negative and a positive control of *C. abortus* DNA were included in each test. Polymerase chain reaction products were visualized by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and examined under UV transillumination.

Polymerase chain reaction products were then purified using a QIAquick Spin PCR purification Kit (QUIAGEN, Hilden, Germany) and sequenced using a DNA sequencing kit (dRhodamine Terminator cycle sequencing ready reaction; Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. All sequences were compared with those of the *Chlamydiae* present in the GenBank database using the BLAST search tool. Attempts to cultivate and isolate the strain were made from each positive sample. An aliquot of one microliter of purified homogenates was inoculated in flasks of McCoy cells (ECACC 90010305), a heteroploid mouse fibroblast line. Infected McCoy cells were grown in MEM (Minimum Essential modified Medium, GibcoBRL, Grand Island, NY, USA) containing 10% heat-inactivated foetal bovine serum (FBS, GibcoBRL, Grand Island, NY, USA) and cycloheximide (2µg/ml) at 37°C with 5% CO_2. When a successful isolation was identified on the chamber slide, the infected cells were passaged by trypsinization into a 25-cm^2 tissue culture flask. Bacteria were harvested after direct immunofluorescence test was heavily positive, and total genomic DNA was extracted from the culture using a QIAamp tissue kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Two hundred microliters of elution buffer were used to re-suspend each extracted DNA sample. The genomic DNA was then stored at -20 °C until further processing.

Three placental tissues (3/40), resulted positive for chlamydial DNA by PCR. Sequence analyses of the positive samples showed 100% similarity with 320-bp putative outer membrane protein region of *C. abortus* (GenBank accession number: EU326104). After 5 days, 2 of the 3 cultures that were infected with placental tissue suspensions, showed the presence of chlamydial-like organisms when tested

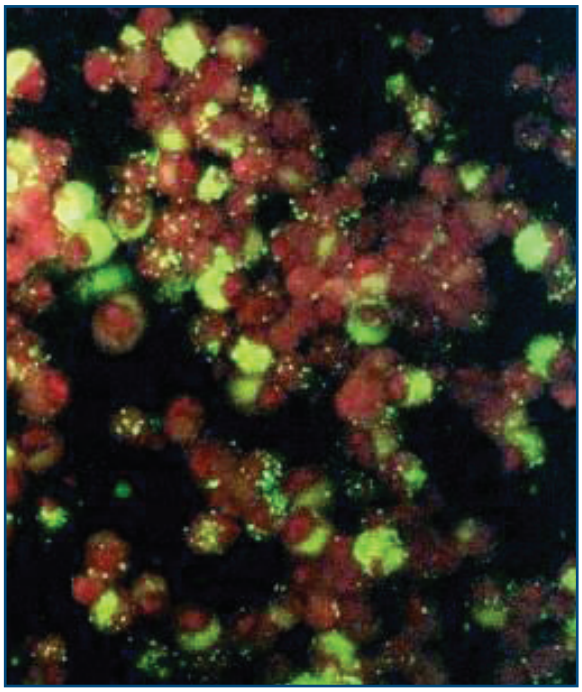

Figure 1. *Presence of chlamydial-like organisms after IFD analysis in McCoy cultures that were infected with 1ml of suspensions of placental tissues*.

by IFD (Figure 1). Moreover, chlamydial DNA was confirmed by PCR analyses. Two *Chlamydia* isolates have been stably maintained in McCoy cells at 37°C as a persistent infection by changing the cell culture medium every 10 days to 2 weeks.

This study documents the first isolation of *C. abortus* from ovine placental tissues in Sardinia. In the authors' previous studies the prevalence of abortion and the role of different infectious agent in abortion were investigated. From 1999 to 2005, 12/213 placentae from sheep resulted positive to *C. abortus* after PCR (4, 5). *C. abortus* is a pathogen of significant economic importance and represents principally a pathogen of ruminants. Sheep can become infected at any age and during any season, but the highest period of risk is undoubtedly at lambing time. Infectious ovine abortion is a major health problem in sheep worldwide, having significant financial and welfare implications. For this reason, an early and accurate diagnosis of the cause of abortion is important, so that appropriate control measures can be adopted to limit or prevent the spread of infection. DNA detection is more rapid and economic than isolation and it can be considered a useful technique for the diagnosis of the previously mentioned pathogens (1). Nevertheless, the isolation of these pathogens from aborted samples represents the gold standard for definitive diagnosis and for the charaterizating of isolated strains. However, isolation requires obtaining samples in optimal conditions (they must be fresh, with little or no contamination, and free of toxic factors), containing a threshold number of live and viable microorganisms. In fact, contamination with other bacteria, inadequate transport conditions, autolysis, and other factors may all adversely affect isolation. Samples should be placed in chlamydia transport medium (SPG) before being despatched to laboratory in order to prevent contaminations.

If the diagnosis is confirmed rapidly and reliably, then control measures can be implemented to reduce the impact of infection in current and future years, thus protecting the economic viability of the flocks. As the disease is commonly transmitted within flocks by infected stock, controls performed at farms level may be beneficial. Farmers will implement control measures where the cost of action is lower than the benefits. Therefore knowledge of the costs and benefits is required in order to support the uptake of disease control strategies.

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