Chronic endometritis in subfertile mares with presence of *Chlamydial* DNA

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Abstract

When endometritis becomes chronic in mares, infertility can follow. Among various causative agents, many bacteria are involved and mono- or mixed-infections are common. In our study, fifty mares with a previous history of subfertility were subjected to clinical and ultrasonographic examination of the reproductive tract, and samples were collected for cytology, bacteriology and PCR for *Chlamydia spp* detection. The aim of this work was to highlight the presence of *Chlamydia abortus* in chronic endometritis of subfertile mares. Endometrial chronic lesions were detected in five of six Chlamydia-positive animals.

Keywords: mare subfertility, chronic endometritis, *Chlamydia spp.*

1. Introduction

Chlamydia abortus is an obligate intracellular gram-negative bacterium that infects a large number of mammalian species, is known to be the agent of the Enzootic Ovine Abortion, but an important and subtle role is represented by its involvement in genital tract infections of the bovine species, causing metritis and infertility [1]. Currently, Sachse et al. adopt the classification that sees the eleven Chlamydia species enclosed in a single genus, the genus Chlamydia [2]. Genital infection, occasional abortion and conjunctivitis have been reported in mares but the relationship between abortion and chlamydial infection is still under discussion [3]. Regarding the involvement of microorganisms belonging to the genus Chlamydia in human infertility, *Chlamydia trachomatis* is one of the main agents involved in PID (Pelvic Inflammatory Disease) and can determine chronic endometritis [4]. Chronic damages due to the persistence of *C. abortus* infection appear to be similar to the lesions found in chronic infection by *C. trachomatis* [5] and similar, in histological aspects, to ocular lesions that are found in Trachoma [6].

Dealing with this theme, a particular attention should be paid to the mare's chronic endometritis (CE). CE often follows the physiological “post breeding endometritis”, that is a common reaction in response to the spermatozoa introduced into the uterus, or it follows repeated artificial inseminations or intrauterine treatments. Microorganisms ascending from the lower genital tract can colonize the uterine cavity; however, to restrict bacterial proliferation and invasion [7,8] mechanisms such as cervical mucus plug, the endometrial epithelium and its immune cellular components (neutrophils, macrophages, and natural killer cells), and elements of the innate immune system, including natural antimicrobial peptides seem to play an important role into eradication of microbial invasions, in some cases this does not happen and we assist at the establishment of CE. Although CE can be asymptomatic, recent studies have shown that it is related with repeated implantation failures after in vitro fertilization-embryo transfer, unexplained infertility, and recurring abortions. CE consists in the protraction of an inflammatory condition of uterine

endometrium characterized by an abnormal pattern of lymphocyte subsets and, consequently, an aberrant endometrial microenvironment [9].

The lack of clearness (precision) in identifying a convincing cause of infertility in observed mares, the attention to the involvement of *Chlamydia abortus* in infertility in course of non species-specific infection and the presence of sheep (reservoire for *C. abortus*) on the grounds where horses were housed, have made us to consider among the various etiopathogenetic hypotheses the presence of *Chlamydia abortus.*

The aim of this work was to highlight the presence of *Chlamydia spp* in chronic endometritis of infertile mares.

2. Materials and methods

This study included fifty mares of various breeds, aged from 4 to 20 years, with mean age ±SD of 12.1±4.0 years, with a previous history of infertility or subfertility, embryonal resorption, abortion. They were housed in paddocks located in the area of Turin (Italy). Their reproductive tract was evaluated by transrectal palpation and ultrasound examination (MyLab^{TM}30Gold, Esaote, Italy), and by vaginal speculum examination. Samples for cytological and bacteriological exams and for DNA detection were collected from all the animals. In twelve cases, when the procedure could be done in relation to the breeding season, also uterine biopsies for histological exams were obtained. Almost all the mares had conformational abnormalities but a Caslick suture had been done to prevent ascending infections of the uterus.

All samples were collected after disinfection of the vulva and perineal area with povidone iodine (Betadine®, MEDA Pharma S.p.A., Milan, Italy). All instruments were passed through the vagina and cervix into the uterus with a sterile sleeved and sterile lubricated arm and all samples were collected from the base of the uterine horns.

A commercial uterine cytological brush (Cytobrush, Minitube, GmbH, Germany) was used to take samples for cytology and DNA. For cytology, the brush was rolled on a glass slide while the brush for DNA was placed in a 5 ml sterile plastic tube (Sigma-Aldrich, Milano, Italy).

A double-guarded cotton swab (Minitube, GmbH, Germany) was used for bacteriological exams and placed in Amies medium (Copan Italia, Brescia, Italy). Uterine biopsies were collected using sterilized uterine biopsy forceps (Equivet, Kruuse, Marselv, Denmark) and placed in 10% tamponed formalin.

The cell smears were fixed and stained using Diff Quick stain (Medion Diagnostics AG, Düdingen, Switzerland), following a routinary procedure [10]. Ten microscopic fields were examined (600X magnification) and the number of PMNs was recorded and interpreted according to the classification of Le Blanc [11].

To demonstrate the chlamydial presence in cytobrushes a nested-PCR based on *ompA* gene [12], followed by DNA sequencing, was performed. Briefly, a DNA extraction kit (Qiagen GmbH, Hilden, Germany) was used to extract DNA from each sample, in according to the manufacturer's instructions. Two sets of primers based on *ompA* gene were used for the first and second step. A strain of *C. psittaci* was used as a positive control in the PCR. The positive amplicons were purified (Affymetrix™ ExoSAP-IT™, USB, Cleveland, Ohio, USA) and sequenced by a commercial resource. Finally, the chlamydia species were identified by NCBI-BLAST (http://www.ncbi.nlm.nih.gov) search of nucleotide sequences.

Microbiological examination was performed using a standard technique [13]. Endometrial swabs were cultured on blood and MacConkey agar plates (Thermo Scientific™ Oxoid, Italy) and incubated for 48h. Miniaturized bacterial identification methods for Gram negative and positive bacteria, respectively, BD BBL Crystal enteric/non fermenter ID kit and BD BBL Crystal Gram-positive ID kit (Thermo Scientific, Italy) were carried out.

Formalin fixed biopsy were paraffin embedded; sections were then Haematoxylin and Eosin stained, according to standard procedure. Histological observation was mainly focused on evidence of increased stromal density, pleomorphic inflammatory infiltrate dominated by lymphocytes and plasma cells, superficial stromal edema, following the classification of Kenney, revised in 1986,

which sees category II, which most of our cases fall into, subdivided into IIa and IIb with reference to various parameters including the degree of fibrosis present [14].

Chlamydia-positive mares were treated with intrauterine oxytetracycline (Panterramicina®, Zoetis Italia Srl) administered in estrous (6g for 3 days, meaning 200ml/die).

Subjects, during first estrus after treatment, were retested for DNA detection following the same procedure descripted before (cytobrush, swab, PCR) and inseminated.

The study was performed in accordance with the guidelines for the care and use of animals of the Department of Veterinary Science of the University of Turin, Italy.

3. Results

Neither clinical nor ultrasound examination of mares revealed any sign of endometritis.

Cytological exams revealed mild endometritis in twenty-four mares, moderate in three and severe in eight ones. In fifteen animals no PMN_S were detected, no Chlamydia inclusion bodies were detected in the samples.

Eleven out of twelve uterine biopsies showed histological traits compatible with grade IIa endometritis, mild to moderate inflammation of the endometrium and/or multifocal areas of periglandular fibrosis. The inflammatory infiltrate was predominantly characterized by lymphocytes. Although the finding of a few of these may be compatible with a normal uterus, even a slight increase may be diagnosed as indicative of chronic endometritis. One case, showed a considerable number of siderocyte. The evidence was probably due to previous hemorrhages. The findings of histological evaluation were in agreement with cytological results.

C. abortus DNA was detected in six samples, one with no-lesions evidenced by cytology, four ones showing a mild chronic endometritis and another one a moderate chronic endometritis (Table 1).

The histological findings of two of the four mild endometritis cases showed different degrees of mononuclear infiltrate and slight desquamation of epithelia (Type IIa) (Fig 1).

Only two out of fifty endometrial swabs resulted positive to bacteriological culture. In the first sample *Enterococcus faecalis* was isolated and in the second one *Staphylococcus epidermidis*. Both culture-positive mares were Chlamydia-positive.

Four of Chlamydia-positive mares were treated in the same breeding season, resulting then Chlamydia-negative at PCR-retest and conceived following Artificial Insemination.

4. Discussion

Our data highlight the presence of *Chlamydia abortus* in subfertile mares affected by chronic endometrial inflammation.

Reproductive anatomy, defective myometrial contractility, lowered immune defences, overproduction of mucus, inadequate lymphatic drainage, or a combination of these factors will predispose the mare to the persistence of post-breeding endometritis [8], leading to CE. In our work, most of examined subjects presented Caslick suture because of the conformational abnormalities. Three mares also showed acquired cervical fibrosis and then uterine fluid accumulation for clearance failure.

Even in recent studies on women's fertility, the role of CE is getting more attention. CE in women can be asymptomatic, it is found in up to 40% of infertile patients and is responsible for repeated implantation failure and recurrent miscarriage [15]. The histological pattern of human CE is characterised by an abnormal expression of lymphocyte subsets and, consequently, an aberrant endometrial microenvironment, which play a critical role in endometrial receptivity [16]. Bacteria involved in equine endometritis are for the most part considered to be opportunistic pathogens. Although the bacterial equine endometritis often shows monoinfection, mixed infections do occur [8]. Chlamydiae have been referred to numerous of diseases in horse among which the most important clinical aspects concern abortion and respiratory tract diseases, although epidemiological and pathological aspects of the disease, as for classification of *Chlamydia spp.* involved remain still unclear. Certainly, the species most involved in horse infections are *C. psittaci [17]* and *C. pneumoniae [18]*, the first related to infections contracted by psittacides and the other, controversial,

it may remain for long time in the respiratory tract of horses with or without symptoms and be transmitted by air flows and genital spreading, determine abortion in pregnant mares and, perhaps, hesitate in capillary aspects such as infertility as peripheral phenomenon. *Chlamydia abortus* is well established as genitopathogenic agent in small ruminants which are the primary reservoir hosts for this organism. Its role in infertility can somehow reflects similarities with *Chlamydia trachomatis* lower genital tract infection in humans, pathogen involved in PID. The clinical spectrum of chlamydial PID ranges from subclinical endometritis to frank salpingitis, tubo-ovarian masses, pelvic peritonitis, periappendicitis and perihepatitis. However, symptomatic chlamydial infections represent only the tip of the iceberg of all chlamydial infections, as the majority of genital chlamydial infections are asymptomatic [19]. On the basis of these considerations we have chosen to investigate the presence of Chlamydia in our subjects. Chlamydiae are specialized in maintaining a long-term relationship with its hosts, modulating and evading the immune system, this avoids the manifestation of markedly evident lesions, except in cases of epicrisis such as abortion While when we are dealing with abortion, a consequence of impairment of the whole maternal organism often with evident macroscopic lesions, the aspects related to infertility are less evident and the result of previous infections that do not allow the detection of M.O. Wittembrick [18] did not found a significant correlation between the detection of uterine Chlamydial infection and clinical sign, but there was a significant association of genital Chlamydial infection and mares that were mated but were not pregnant. In our piece of work, three out of six Chl-positive mares were empty from more than two years and three manifested recurrent abortions or embryo reabsorptions. Although Chlamydia positive samples were in a small number, it seems that these are the ones with the mildest lesions both on histopathology and cytology. In these samples, there is almost a very low degree of fibrosis and the most focal aspect of the lymphocyte infiltrate. This event could suggest that the infection had occurred long ago and that now only the presence of the DNA of the microorganism remains detectable. The same *C. trachomatis* is able to induce subtle chronic inflammation where the M.O, in its integrity, it is no longer found but its DNA remains indelible for

a long time. This is one of the motivations, in addition to the sensitivity and ease of finding sample that have made DNA detection method so famous in Chlamydial diagnostic protocol. On the basis of cytological and histological findings and the fact that flocks of sheep were transited on the land where the mares were housing we considered it appropriate to verify the presence of this microorganism or traces of this by use of PCR followed by sequencing.

5. Conclusions

Based on these considerations and on our results, we can point out that *C. abortus* may play a role in mare's infertility, alone or in co-presence with other microorganisms. Its possible role in causing CE can be worth being investigated, since its presence can somehow induce endometrial chronic damage, even if mild.

After having done all the standard tests without having got a diagnosis, it could be worth testing also for Chlamydial DNA through PCR that can be done from cytobrush samples.

Our adopted PCR protocol is able to detect small amount of chlamydial DNA from collected smears, is not invasive and, at present, it is not particularly expensive (61€ at the University Veterinary Hospital of Turin), therefore, in the light of our results, we would like to recommend its execution, if not for all the hypofertile mares, certainly for those in which it was not possible arrive to a proper diagnosis by other diagnostic tests (bacteriological, cytological, biopsy), which showed mild endometritis at cytological and histological examination, and residing in places of potential sheep grazing.

At the end of that, in case of detection of *C. abortus* in infertile mares, intrauterine oxytetracycline administration may represent an option to increase the possibility of pregnancy. Our results show that mares with CE and Chlamydia-positive findings conceived and maintained pregnancy after appropriate antibiotic treatment.

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