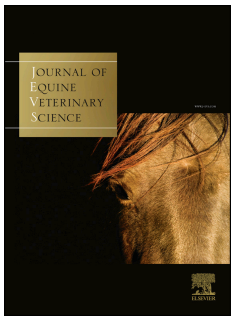
Accepted Manuscript



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

Tiziana Nervo, Patrizia Nebbia, Alessia Bertero, Patrizia Robino, Maria Cristina Stella, Ada Rota, Simonetta Appino

PII: S0737-0806(18)30672-5

DOI: https://doi.org/10.1016/j.jevs.2018.12.003

Reference: YJEVS 2640

To appear in: *Journal of Equine Veterinary Science*

Received Date: 18 October 2018

Revised Date: 23 November 2018

Accepted Date: 6 December 2018

Please cite this article as: Nervo T, Nebbia P, Bertero A, Robino P, Stella MC, Rota A, Appino S, Chronic endometritis in subfertile mares with presence of *Chlamydial* DNA, *Journal of Equine Veterinary Science* (2019), doi: https://doi.org/10.1016/j.jevs.2018.12.003.

Dear Editor,

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

Simonetta Appino

Torino, 10/18/2018

prof. Simonetta Appino, DVM, PhD

Dipartimento di Medicina Veterinaria

Via Vienna 2, 07100 Sassari

Università degli Studi di Sassari

simo@uniss.it

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

Tiziana Nervo[a], Patrizia Nebbia[a], Alessia Bertero[c], Patrizia Robino[a], Maria Cristina Stella[a], Ada Rota[a], Simonetta Appino[b*]

[a] Department of Veterinary Sciences, University of Turin, Largo Paolo Braccini 2-5, 10095 Grugliasco, Italy

[b*] Department of Veterinary Medicine, University of Sassari, via Vienna 2, 07100 Sassari, Italy

e mail: simo@uniss.it

[c] Department of Veterinary Medicine, University of Milan, via dell'Università 6, 26900, Lodi, Italy

*corresponding author: Simonetta Appino, simo@uniss.it

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, histology, 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. It 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 groups the eleven Chlamydia species 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]. Microorganisms belonging to the genus Chlamydia play a role 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 *Chlamydia 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 subfertility in mares, a particular attention should be paid to chronic endometritis (CE). CE often follows “post breeding endometritis”, that is a common reaction in response to semen introduction into the uterus, or follows repeated artificial inseminations or intrauterine treatments. Microorganisms ascending from the lower genital tract can colonize the uterine cavity; in normal conditions, 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 to restrict bacterial proliferation and invasion [7,8]. When defence mechanisms are ineffective or conformation anomalies impair uterine clearance, we assist at the establishment of CE. 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. 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. [9].

The impossibility to identify a convincing cause of infertility, the attention at the involvement of *Chlamydia abortus* in infertility in non species-specific infection, and the presence of sheep (reservoire for *C. abortus*) on the grounds where the mares were housed, led us to consider the presence of this microorganism among the various etiopathogenetic hypotheses.

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, with mean age ±SD of 12.1±4.0 years, (range 4-20 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, ultrasound (MyLab^{TM}30Gold, Esaote, Italy) and 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 histology were obtained. Almost all the mares had conformational abnormalities but a Caslick suture had been placed to prevent ascending infections of the uterus.

The vulva and perineal area were disinfected with povidone iodine (Betadine$^{®}$, MEDA Pharma S.p.A., Milan, Italy) and all the instruments were passed through the vagina and cervix into the uterus with a sterile sleeved and sterile lubricated arm. 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% buffered 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 in accordance with the classification of Le Blanc [11].

To demonstrate the presence of Chlamydial DNA 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 (ExoSAP-IT™, USB, USB, Cleveland, 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 ((Beck. Dick. Comp., Maryland, USA) 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. The classification of Kenney, revised in 1986, in which

category II is subdivided into “a” and “b” with reference to various parameters including the degree of fibrosis, was used [14].

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

During the first estrus after treatment, the mares were retested for DNA detection (same procedure as 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 the mares revealed any sign of endometritis.

Cytology showed mild endometritis in twenty-four animals, moderate in three and severe in eight ones. In fifteen animals no PMN_S were detected. Chlamydia inclusion bodies were never 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. In a case a considerable number of siderocyte was observed, probably due to previous hemorrhages. Histological results were in agreement with cytological findings.

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 Chlamydia-positive mares were treated in the same breeding season, resulting 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. Most of the mares included in our study had a Caslick suture done because of conformational abnormalities, thus preventing ascending contamination of the uterus. 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 diseases in horses, among which the most important clinical aspects concern abortion and respiratory tract diseases, although the epidemiological and pathological aspects of the diseases and the responsible Chlamydial species remain still unclear. Certainly in horse infections, the most involved species are *C. psittaci [17]* and *C. pneumoniae [18]*, the first one related to infections contracted by psittacides while the other is 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 route, determine abortion in pregnant mares

and, perhaps, hesitate in capillary aspects such as infertility as a 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 reflect the role of *Chlamydia trachomatis* in lower genital tract infections in humans, a 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, thus avoiding the manifestation of markedly evident lesions, except in cases of epicrisis such as abortion. While dealing with abortion often evident macroscopic lesions are present, the aspects related to infertility are less evident and may represent the result of previous infections that do not allow the detection of the microorganism. 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 work, three out of six Chlamydia-positive mares were empty since more than two years and three ones showed recurrent abortions or embryo reabsorptions. Although in a small number, Chlamydia-positive samples seemed to be the ones that showed the mildest lesions both on histopathology and cytology. In these samples, there is always 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 microorganism, in its integrity, it is no longer found, but its DNA remains indelible for a long time. On the basis of cytological and histological findings and the fact that flocks of sheep had passed

through the fields where the mares were housed, we considered it appropriate to verify the presence of this microorganism or traces of it.

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.

We can suggest that, in case the standard tests have not led to a diagnosis, it could be worth testing also for Chlamydial DNA through PCR, a search that that can be done from cytobrush samples, especially when the history tells of a possible contact with sheep.

In case of detection of *C. abortus* in infertile mares, intrauterine oxytetracycline administration may represent an option to increase the possibility of pregnancy.

The work was funded by the Italian Ministero dell'Istruzione, Università e Ricerca (ex 60% 2016).

References

[1] Bassan Y, Ayalon N. Abortion in dairy cows inoculated with epizootic bovine abortion agent *(Chlamydia).* Am J Vet Res 1971;32:703-10.

[2] Sachse K, Bavoil PM, Kaltenboeck B, Stephens RS, Kuo CC, Rosselló-Móra R, et al. Emendation of the family *Chlamydiaceae*: proposal of a single genus, *Chlamydia,* to include all currently recognized species. Syst Appl Microbiol 2015;38:99-103.

[3] Rubio-Navarrete I, Montes-de-Oca-Jiménez R, Acosta-Dibarrat J. Prevalence of *Chlamydia abortus* Antibodies in Horses From the Northern State of Mexico and Its Relationship With Domestic Animals. J Equine Vet Science 2017;56:110-3.

[4] Mårdh PA, Møller BR, Ingerselv HJ, Nüssler E, Weström L, Wølner-Hanssen P. Endometritis caused by *Chlamydia trachomatis.* Br J Vener Dis 1981; 57:191-5.

[5] Askienazy-Elbhar M, Suchet JH. Persistent "silent" *Chlamydia trachomatis* female genital tract infections. Infect Dis Obstet Gynecol 1999;7:31-4.

[6] Derrick T, Roberts Ch, Last AR, Burr SE, Holland MJ. Trachoma and ocular Chlamydial infection in the era of genomics. Mediators Inflamm 2015; 2015:791847.

[7] Ferris RA, McCue PM, Borlee GI, Glapa KE, Martin KH, Mangalea MR, et al. Model of chronic equine endometritis involving a *Pseudomonas aeruginosa* biofilm. Infect Immun 2017; 85(12):e00332-17.

[8] Woodward EM, Troedsson MH. Inflammatory mechanisms of endometritis. Equine Vet J 2015;47:384-9.

[9] Matteo M, Cicinelli E, Greco P, Massenzio F, Baldini D, Falagario T, et al. Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. Am J Reprod Immunol 2009; 61:322–9.

[10] Cocchia N, Paciello O, Auletta L, Uccello V, Silvestro L, Mallardo K, et al. Comparison of the cytobrush, cottonswab, and low-volume uterine Flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares. Theriogenology 2012;77:89–98.

[11] LeBlanc MM. Uterine cytology. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. 2nd ed. Wiley-Blackwell; 2011, p. 1922–8.

[12] Cahota R, Ogawa H, Mitsuhashi Y, Ohya K, Yamaguchi T, Fukushi H. Genetic diversity and epizootiology of *Chlamydophila psittac*i prevalent among the captive and feral avian species based on VD2 region of ompA gene. Microbiol Immunol 2006;50:63-78.

[13] Jorgensen JH, Pfaller MA, Carroll KC, Funke G, Landry ML, Richter SS, et al. Manual of clinical microbiology, Eleventh Edition. Washington DC, ASM Press 2015.

[14] Kenney RM, Doig PA. Equine endometrial biopsy. In: Morrow DA editor. Current Therapy in Theriogenology. Philadelphia, WB Saunders; 1986, p. 723–9.

[15] Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. Reprod Sci 2014;21:640-7.

[16] Moreno I, Cicinelli E, Garcia-Grau I, Gonzalez-Monfort M, Bau D, Vilella F, et al. The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology. Am J Obstet Gynecol 2018;218:602.

[17] Jenkins C, Jelocnik M, Micallef ML, et al. An epizootic of *Chlamydia psittaci* equine reproductive loss associated with suspected spillover from native Australian parrots. Emerg Microbes Infect 2018;7:88.

[18] Wittenbrink MM. Aetiological significance of chlamydial infections in equine reproductive disorders. Pferdeheilkunde 1999;15:538-41.

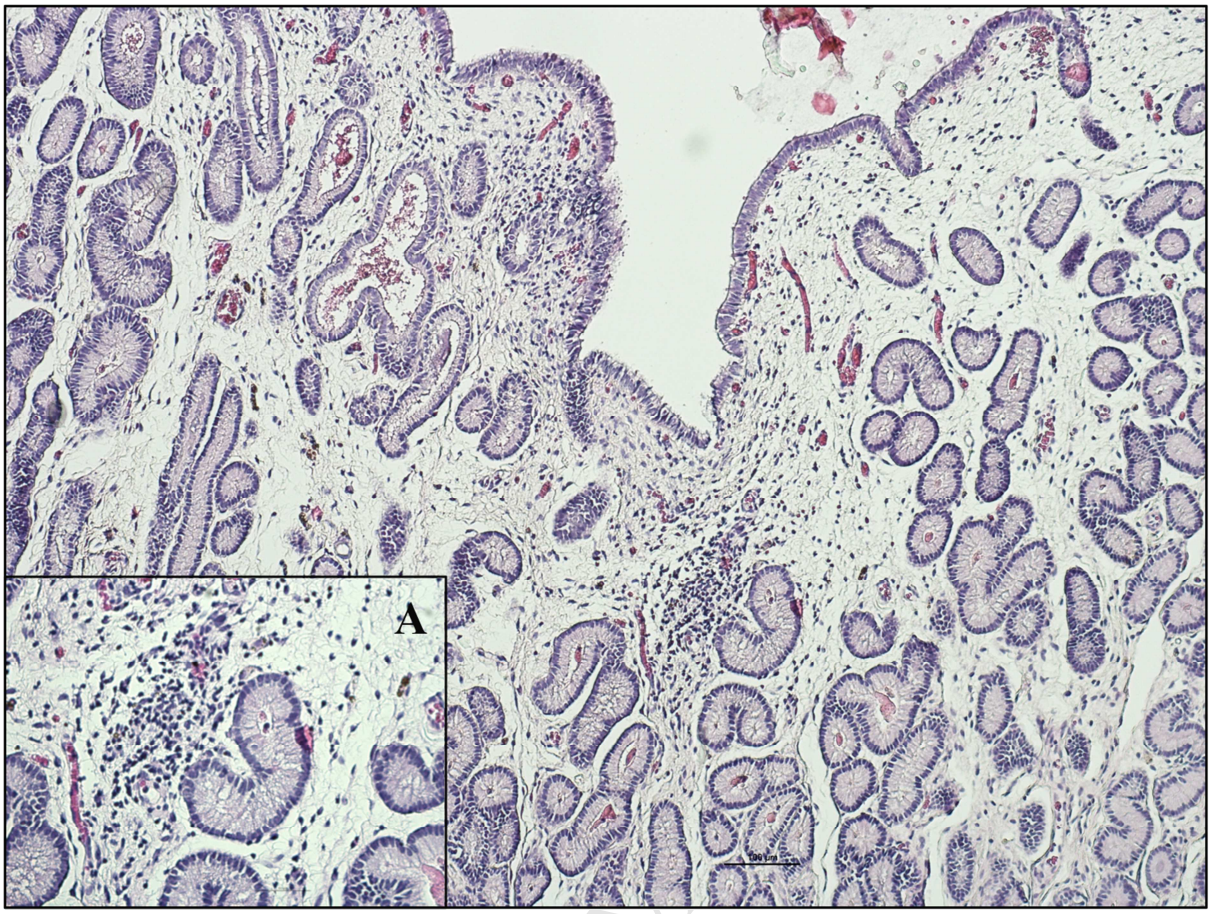
[19] Malhotra M, Sood S, Mukherjee A, Muralidhar S, Bala M. Genital *Chlamydia trachomatis*: An update. Indian J Med Res 2013;138:303-16.

N	BREED	AGE (Yrs)	HISTORY	CULTURE	CYTOLOGY*	BIOPSY§	TREATMENT	POST TREATMENT PCR CHLAMYDIA	PREGNANCY
1	Pony	>20	Empty (> 2 seasons)	Neg	Normal	**2A** (mild focal infiltr lnf in the *spongiosum* layer)	NO	/	/
2	Standardbred	11	Embryo resorptions	Neg	Mild endometritis	/	YES	NEG	YES
3	Standardbred	15	Abortions Red Bag last pregnancy	*Enterococcus spp*	Mild endometritis	**2A** (mild fibrosis, mild lnf moder. siderocytes, *spongiosum* layer)	YES	NEG	YES
4	Standardbred	11	Empty (> 2 seasons)	Neg	Moderate endometritis	/	YES	NEG	YES
5	Thoroughbred	11	Embryo resorptions	Neg	Mild endometritis	/	NO	/	/
6	Standardbred	13	Empty (> 2 seasons)	*Staphylococcus epidermidis*	Mild endometritis	/	YES	NEG	YES

* Mild endometritis (0-2 PMN/field) - Moderate (3-5 PMN/field) - Severe (>5PMN/field) [10]
§ [14]

Table 1 Culture, cytology and histology results of the six Chlamydia-positive horses and breeding outcome following treatment

Fig. 1 uterine biopsy: E.E. stain, 10X, mild focal mononuclear infiltrate; A, 40X higher magnification showing of periglandular infiltrate



Highlights:

- chronic endometritis can be present in subfertile mares
- *Chlamydia abortus* is present in mares with subfertility problems
- there can be an association between Chlamydial DNA and chronic endometritis in mares
- in case of Chlamydial DNA detection in subfertile mares, intrauterine oxytetracycline administration may represent an option to increase the possibility of pregnancy

Tiziana Nervo , project administration

Patrizia Nebbia , methodology in microbiology

Alessia Bertero , methodology in sampling

Patrizia Robino , methodology in microbiology

Maria Cristina Stella , methodology in microbiology

Ada Rota , funding acquisition, supervision,writing – review & editing.

Simonetta Appino, visualization; pathologist ; scientific supervision, writing