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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1684836> since 2019-01-09T11:07:36Z

Published version:

DOI:10.1016/j.jevs.2018.12.003

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

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13 ***Abstract***

14

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

22

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

24

25 **1. Introduction**

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

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

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

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

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

59 **2. Materials and methods**

60 This study included fifty mares of various breeds, aged from 4 to 20 years, with mean age \pm SD of
61 12.1 ± 4.0 years, with a previous history of infertility or subfertility, embryonal resorption, abortion.

62 They were housed in paddocks located in the area of Turin (Italy). Their reproductive tract was
63 evaluated by transrectal palpation and ultrasound examination (MyLabTM30Gold, Esaote, Italy), and
64 by vaginal speculum examination. Samples for cytological and bacteriological exams and for DNA
65 detection were collected from all the animals. In twelve cases, when the procedure could be done in
66 relation to the breeding season, also uterine biopsies for histological exams were obtained. Almost
67 all the mares had conformational abnormalities but a Caslick suture had been done to prevent
68 ascending infections of the uterus.

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

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

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

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

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

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

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

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

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

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

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

109 **3. Results**

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

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

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

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

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

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

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

130 **4. Discussion**

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

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

139 Even in recent studies on women's fertility, the role of CE is getting more attention. CE in women
140 can be asymptomatic, it is found in up to 40% of infertile patients and is responsible for repeated
141 implantation failure and recurrent miscarriage [15]. The histological pattern of human CE is
142 characterised by an abnormal expression of lymphocyte subsets and, consequently, an aberrant
143 endometrial microenvironment, which play a critical role in endometrial receptivity [16]. Bacteria
144 involved in equine endometritis are for the most part considered to be opportunistic pathogens.
145 Although the bacterial equine endometritis often shows monoinfection, mixed infections do occur
146 [8]. Chlamydiae have been referred to numerous of diseases in horse among which the most
147 important clinical aspects concern abortion and respiratory tract diseases, although epidemiological
148 and pathological aspects of the disease, as for classification of *Chlamydia spp.* involved remain still
149 unclear. Certainly, the species most involved in horse infections are *C. psittaci* [17] and *C.*
150 *pneumoniae* [18], the first related to infections contracted by psittacides and the other, controversial,

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

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

182 **5. Conclusions**

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

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

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

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

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

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