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Reproductive disorders induced by *Chlamydophila* spp. infections in an italian mediterranean buffalo (*bubalus bubalis*) herd

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ABSTRACT: The Italian Mediterranean Buffalo (*Bubalus bubalis*) has low fecundity and high incidence of abortion. Several studies have associated reproductive failure of water buffalo with viral infections but there is limited information on the role of chlamydial infections. To investigate the presence and the role of *Chlamydiaceae* in water buffalo a retrospective study was performed in a farm where, in the arch of 11 months, the pregnant heifers suffered an abortion rate of 36.8% in the 3rd and 5th month of pregnancy. Antibodies to *Chlamydiaceae* were detected in 57% of the aborted cows, while the rate of positivity was 0% in overtly healthy cows used as control. By a PCR assay 3 of 14 vaginal swabs from aborted animals tested positive for *Chlamydophila* agents and, additionally, 3 out of 7 aborted foetuses tested positive for *Chlamydophila* spp., with two being co-infections by *Cp. abortus* and *Cp. pecorum* and one being characterised as *Cp. abortus*. The presence of anti-*Chlamydiaceae* antibodies in 57% of the aborted animals and the detection of *Chlamydophila* agents in foetal organs and in vaginal swabs is consistent with the history of abortions ($P<0.002$) observed in the herd and may suggest a pathogenic role by *Chlamydophila* spp. in water buffalo.

Key words: Water buffalo, *Chlamydophila* spp., Abortion.

INTRODUCTION - The Italian Mediterranean Buffalo (*Bubalus bubalis*) is an indigenous breed of water buffalo and is mainly reared in the south-western regions of Italy. The species is particularly appreciated for the production of the worldwide known "mozzarella" cheese but is affected by low-rate reproductive efficiency. Its reproduction is under the influence of seasonal changes in the day-light duration tending to display low rates of pregnancy with increasing photoperiod (Baruselli *et al*., 1997). In the animals inseminated both naturally and artificially from mid-winter to summer, the rates of successful pregnancy are low due to frequent embryonic mortality and abortion (Zicarelli *et al*., 1997; Campanile *et*

al., 2005). Moreover, several studies have associated reproductive failures of water buffalo with viral infections such as BoHV.1 or BoHV.4 (Pinto *et al*., 2003; Dewals *et al*., 2006), but there is limited information on the role of chlamydial infections which have associated with several reproductive diseases of cattle (Storz *et al.,* 1960). *Chlamydophila* (*Cp.) abortus* and/or *Cp. pecorum* may be related to abortion at different stages of pregnancy, metritis, salpingitis, repeat breeders and mastitis (Cavirani *et al.,* 2001; DeGraves *et al.,* 2003). Due to the close species affinity between water buffaloes and bovines and to the low host specificity exhibited by the various chlamydial species and strains, it may be hypothesized that chlamydial bacteria may also be implicated in reproductive disorders of buffaloes. In this study the presence and the pathogenic role of 2 different C*hlamydophila* species is described in an Italian Mediterranean Buffalo herd with a long-term history of abortion.

MATERIAL AND METHODS - From April 2005 to February 2006 a total of 25 cases of abortion occurred in pregnant heifers at the 3rd-5th month of gestation in a water buffalo (*Bubalus bubalis*) herd of Italian Mediterranean Buffalo breed consisting of 165 lactating cows, 168 dry cows and 68 pregnant heifers. Abortions occurred in a scattered fashion throughout the 11-months period, with increased frequency in August (7/25), November (5/25), January (4/25) and February (3/25). **Study design and samples collection.**- One month after the last episode of abortion, a case-control study was performed on 28 buffaloes selected by a systematic stratified sampling method at the 95% Confidence Interval (CI). Four-teen animals (#1 to 14) were cows that had aborted their first pregnancy. Seven such cows were selected because the aborted foetus (#8 to 14) was also available. Four-teen healthy animals (animals # 15 to 28) from the same herd were used as control. From all the buffaloes included in the study, serum samples and vaginal swabs were collected. The vaginal swabs were stored at -20°C before being submitted for microbiological investigations. Seven foetuses (#8 to 14) from 7 of the 25 cases of abortion were available for diagnostic examinations, as they had been collected and frozen at -20°C. The foetuses were assayed for viral, chlamydial and other abortive bacterial agents. Due to improper storage, the other 18 foetuses were not analysed. **Bacteriological investigations -** Specimens from lungs, liver, spleen and kidneys of the foetuses were screened for *Brucella* spp. and *Salmonella* spp. infections by using cultural methods. Since for the isolation of chlamydial agents by biological samples require appropriate storage at temperatures not higher than -80°C, attempts to cultivate these pathogens were not made and the diagnosis was based on molecular assays. **Virological assays** - Foetal tissue samples and vaginal swabs were screened for Bovid herpesvirus 1 (BoHV.1), Bovid herpesvirus 4 (BoHV.4) and bovine viral diarrhoea virus (BVDV) by using 3 distinct PCR assays. **For detecting bacteria belonging to *Chlamydiaceae* family** the samples were analysed by using a nested PCR procedure that targets the *omp*A gene, described by Kaltenböck *et al.* (1997) and modified by Sachse and Hotzel (2002). In the first-step (family-specific) PCR, the set of primers 191CHOMP (5'-GCI YTI TGG GAR TGY GGI TGY GCI AC-3') and CHOMP371 (5'-TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC-3') yields amplicons of 600 bp in length for all of the species belonging to the *Chlamydiaceae* family. One µl of the amplicon was used as template for the second-round amplification, using the primer set 218CHOMP (sense) (5'-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3') / CHOMP336 (antisense) (5'-CCR CAA GMT TTT CTR GAY TTC AWY TTG TTR AT-3'), that yields a 400 bp product for both closely related *Cp. abortus* and *Cp.*

psittaci species, and the primer set 204CHOMP (sense) (5'-CCA ATA YGC ACA ATC KAA ACC TCG C -3') / CHOMP336 (antisense), that yields a 440 bp product for *Cp. pecorum*. ***Sequence analysis –*** To obtain the identification at the specie level the sequences of the second-round PCR amplicons obtained with both primers 218/336 and primers 204/336 from the swabs and fetal tissues were determined by using ABI Big Dye chain-terminating reactions and an automated machine (ABI 377). The sequences were assembled with Bioedit software package version 2.1 (Hall, 1999) and compared to cognate sequences in the genetic databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA (http://www.ebi.ac.uk/fasta33) web-based programs. **Serological assays for *Chlamydiaceae* antibodies** - All the 28 sera collected from animals (aborted and control) were assayed for the presence of IgG immunoglobulins anti-*Chlamydiaceae* LPS by using an ELISA commercial kit (Chekit-Chlamydia EIA, Bommeli, Liebefeld-Bern). Optical density (OD) was read at 450 nm. The value of each sample was expressed as a percentage of positive reference standard by taking a negative reference serum as the zero value according to approved standardization methods. Serum samples exceeding the threshold value of 40% were considered as positive, according to manufacturer's guidelines. **Statistical methods -** Serological data were cross-tabulated against the abort events and the statistical significance was assessed by using the Fisher's exact test. Relative risk at 95% Confidential Interval (CI) was used to determine the magnitude of the associations. Differences between the optical densities were assessed by the t-Student test.

RESULTS AND CONCLUSIONS - In this study the role of chlamydial bacteria as a causative agent of epizootic abortion in buffaloes was investigated retrospectively. By microbiological investigations no viruses and abortifaciens bacteria were detected in both foetal and vaginal samples. By using the ELISA assay to detect anti-*Chlamydiaceae* antibodies the mean value of the sera of the healthy buffalo cows was 10.19 ± 11.65%, with the highest value of 27.5%. Accordingly the sera were considered negative. Six out of 14 (43%) sera collected from the aborted buffalos also tested negative, with the mean value of 1.67± 4.08%. By converse, 8 of 14 (57 %) sera collected from the aborted buffalos tested positive with a mean value of 75.18 ± 25.39%. By using the Fischer's exact test, a significant association ($P<0.02$) between seropositivity to *Chlamydiaceae* and the abortion cases occurred in the herd was found, with a Relative Risk of abortion of 3.3 (95% Confidential Interval: 2.23-6.50) in seropositive buffaloes. These serological findings were suggestive of chlamydial infections in aborting cows but more conclusive evidence was obtained by PCR and by sequence analysis results, with the identification of two distinct chlamydial species that were co-circulating in the aborting animals. Three out of 8 seropositive cows (# 1, 12 and 14) were found to shed *Chlamydophila* spp. by the vaginal route while none of the vaginal swabs from the healthy animals tested positive for chlamydial agents. In detail, the vaginal sample from the cow (#) 1 was found infected by *Cp. pecorum*, the vaginal sample # 12 by *Cp. abortus* and the vaginal sample # 14 was co-infected by both *Cp. abortus* and *Cp. pecorum*. Three out of 7 foetuses were found positive by nested-PCR with *Cp. abortus* that was detected in the tissue samples (placenta, lungs, liver, spleen and kidney) of the fetus # 12 and both *Cp. abortus* and *Cp. pecorum* that were detected in the tissues of foetuses # 10 and # 14. Altogether, the findings of this study revealed the presence of *Chlamydophila* spp in an outbreak of abortion in buffaloes, suggesting an abortive role of these pathogens and

stressing the need for adequate plans of surveillance, prevention and control. *Chlamydophila* spp should be always included in diagnostic algorithms in the presence of reproductive disorders, in order to assess the real burden of *Chlamydophila*-associated disease in buffaloes and to understand whether vaccines are required.

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