Short Communication

Retrospective Biomolecular Investigation of *Coxiella burnetii* and *Leptospira* spp. DNA in Cases of Abortion, Stillbirth and Neonatal Mortality in Dogs and Cats

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A B S T R A C T

Abortion and neonatal mortality are events that can occur in breeding bitches and queens. It has been reported that up to 55% and 33% of these cases remain without a known cause, respectively, in canine and feline pregnancies. Unusual abortigenic and potentially zoonotic agents, including *Coxiella burnetii* and *Leptospira* spp., may be involved in these cases. *C. burnetii* is able to cause reproductive disorders in cattle, sheep and goats, and cases of abortion have been observed in dogs and cats. Moreover, several outbreaks of *C. burnetii* infection in humans have been caused by delivering bitches and queens, and some of these animals experienced abortion. *Leptospira interrogans sensu lato* is able to cause abortion or stillbirth in several animal species and its abortigenic role has occasionally been described in bitches and queens. The aim of this study was to search for *C. burnetii* and *Leptospira* spp. DNA in a retrospective series of 103 cases of canine and feline abortion, stillbirth, and neonatal mortality submitted for the identification of possible infectious agents. One hundred and fifty-one specimens were tested using PCR assays and found negative for *C. burnetii* and *Leptospira* DNA. However, in 49 samples (47.6%) other infectious causes of abortion, stillbirth, and neonatal mortality were identified. These results showed that *C. burnetii* and *Leptospira* spp. are probably not common abortigenic agents or causes of neonatal deaths in dogs. However, given the potential abortigentic and zoonotic role of these agents, surveillance of canine and feline abortion, stillbirth, and neonatal mortality could be advisable for a systematic investigation of these events.

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Introduction

Abortion, stillbirth, and neonatal mortality are events that can occur in canine and feline reproductive medicine. Their reported incidence ranges from 5% to 35%. The etiology of these losses is complex and can be classically divided into infectious and noninfectious causes. Among the infectious causes, bacterial diseases are reported as a primary cause of mortality in puppies and kittens during the first week of age.[1] *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, *Brucella canis*, *Campylobacter* spp., and *Salmonella* spp., are described as the most frequently isolated pathogens causing pregnancy losses in bitches and queens and canine and feline neonatal mortality.[2−4]

Canine distemper virus, canine herpesvirus (CHV-1), canine parvovirus (CPV), canine minute virus, feline herpesvirus (FHV-1), feline panleukopenia virus (FPV), and feline infectious peritonitis, caused by a coronavirus, are reported as the most common viral causes of canine and feline pregnancy loss and neonatal mortality. *Toxoplasma gondii* and *Neospora caninum* also are considered as rare causes of abortion and neonatal mortality.[1,5]

Nevertheless, a percentage of these cases of abortion, stillbirth, and neonatal mortality remain without an aetiological identification. For example, it has been reported that 33% of feline neonatal mortality[2] and 55% of canine neonatal deaths[3] were attributed to unknown etiology. In these cases, the involvement of unusual infectious pathogens not routinely investigated in canine and feline abortion, stillbirth, and neonatal mortality cannot be excluded.

Worldwide, *C burnetii* and *Leptospira interrogans sensu lato* are bacterial agents well known for their potential abortigenic role in many animal species. *C. burnetii* is the causative agent of an important and underdiagnosed zoonosis with worldwide distribution, Q fever. Livestock species have traditionally been considered as reservoirs of infection, with microorganisms found in high concentrations in the placenta and reproductive tracts of cattle, sheep, and goats. However, the potential role of other animal reservoirs, including cats and dogs, has been supposed.[6] Antibodies against *C. burnetii* have been detected in the serum of cats worldwide.[7] Moreover, it has been hypothesized that cats have been responsible for transmission of *C. burnetii* to humans in Q fever outbreaks due to exposure to parturient queens, in which various degrees of seroprevalence have been detected.[7−10] Nagaoka et al.[11] provided the first evidence of active infection, isolating 9 of 29 cases of *C. burnetii* from vaginal swabs of domestic queens, and *C. burnetii* DNA was amplified through PCR assay from 3 out of 37 uterine tissues from cats with and without a history of reproductive abnormalities.[12] In canine species, the epidemiological role of *C. burnetii* infection is still debated; however, seropositivity has been reported in dogs, and human outbreaks associated with parturient bitches have been described.[13,14] *C. burnetii* DNA has also been recently detected in 4 of 54 canine placentas originating from aborting animals in The Netherlands.[15]

Leptospirosis is a worldwide re-emerging disease caused by a gram-negative bacterium of the genus *Leptospira*. There are over 250 pathogenic serovars that are adapted to different wild or domestic animal reservoir hosts, and serovars are further grouped into antigenically related serogroups. Infection with pathogenic leptospires can cause a wide range of clinical manifestations from subclinical to severe and potentially lethal disease.[16] However, there are only a few

reports of reproductive disorders in dogs related to leptospiral infection; abortion has occurred in dogs after transplacental spread of the serovar Buenos Aires,[17] and a report suggested that abortion was associated with serovar Bratislava infection.[18] Some commercial companies that promote diagnostic devices currently suggest including leptospirosis testing in cases of abortion in dogs. In feline species, seroprevalence of *Leptospira* spp. varies from 0% to 35% depending on the geographical area and the diagnostic methods used[16]; however, despite serological evidence, there is little information about clinical leptospirosis in cats. A recent report described 3 confirmed and naturally infected clinical cases of feline leptospirosis presenting with renal failure.[19] Stillbirths, abortions, and retained placenta due to *Leptospira* spp. also have been reported in queens.[20]

The aim of this study was to search for *C. burnetii* and *Leptospira* spp. DNA in a retrospective series of cases of canine and feline abortion, stillbirth, and neonatal mortality submitted to a diagnostic laboratory of infectious diseases.

Materials and Methods

Sample Selection

Considering a normal gestation period of 57-72 days for dogs and 52-74 days for cats, the cases were classified as follows: abortion was defined as fetal loss during the second half of pregnancy and characterized by the expulsion of a dead conceptus or a living one incapable of independent life; stillbirth was defined as puppies or kittens reported as dead at birth; neonatal mortality was divided into early neonatal mortality if puppy or kitten death occurred within 7 days after birth and late neonatal mortality if the death occurred 7-28 days after birth.

According to this classification, abortions, stillbirth cases, and dead neonatal dogs and cats were collected between 2008 and 2015 by clinicians specializing in small animal reproduction. The samples were promptly sent to the infectious disease laboratory, Department of Veterinary Medicine (University of Perugia) to search for at least one of the most common infectious agents of abortion or neonatal mortality, both bacterial and viral, including CHV-1 and CPV for dogs and FPV and FHV-1 for cats. The pathological materials arrived at the laboratory within 24 hours of the event (abortion, stillbirth, or neonatal mortality) and were maintained refrigerated at 4°C until processing. When the veterinarians were not able to send the pathological material within 24 hours, it was stored frozen and sent afterward.

While a biomolecular method was used to detect the viral agents (CHV-1 and CPV for dogs; FPV and FHV-1 for cats), a standard bacteriological examination was performed. These examinations were not carried out systematically, but only when required by the referring veterinarian and only when the samples were suitable for the test required (for example, only refrigerated samples for the bacteriological examination). For the bacteriological examination, samples were inoculated onto blood agar, mannitol-salt agar, and MacConkey's agar plates. The plates were incubated for a minimum of 24 hours at 37°C under aerobic and anaerobic conditions. Isolation of a pure or predominant bacterial species from all the specimens originating from the same puppy or kitten, was considered a positive result. The identification of bacteria was performed by examining colony characteristics, gram staining, and use of biochemical tests and commercially available API kits (bioMerieux, Étoile, France). Breed and exposure to risk factors for infectious diseases were not recorded or investigated.

Biomolecular Investigation

DNA extraction from placenta (when available) and a pool of lung, liver, and spleen from the canine or feline foetuses or neonates was performed using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis). The concentration and purity of the extracted DNA were quantified using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan, Italy). The extracted DNA was stored at −20°C until use. A PCR protocol described by Berri et al.[21] amplifying a fragment of 687 bp of the IS*1111* insertion sequence was used to detect *C. burnetii* DNA. The previously reported detection limit of the *C. burnetii* PCR assay was 10^{-2} dilution of *C. burnetii* positive control DNA (comparable to 12 pg/μl of DNA),[12] but considering that the mean number of IS*1111* copies varies between 7 and 110,[22] no exact limit was defined.

A nested PCR protocol for *Leptospira* spp. was developed to increase the sensitivity of a conventional PCR previously described,[23] amplifying a preserved fragment of the 16S rRNA gene present in both saprophytic and pathogenic *Leptospira* spp. The first PCR was performed using the previously published primer pair, resulting in a 330 bp product, whereas an internal pair of primers (FN: 5'-CATG-CAAGTCAAGCGGAGTA-3'; RN: 5'-GGCTCATCTCCGAGCAATAA-3') was designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), yielding a fragment of 166 bp. Serial 10-fold dilutions of the *Leptospira* controls, ranging from 5 ng/μL to 0.05 pg/μL, were carried out to determine the increase in sensitivity of the nested protocol in the biological samples compared with the single protocol. Both saprophytic and pathogenic Leptospirae, kindly provided by the National Reference Centre for Animal Leptospirosis (*L. interrogans* serovar *pomona*, type *Mezzano I*; *L. interrogans* serovar *hardjo,* type *Hardjoprajitno*; *L. interrogans* serovar *Bratislava,* type *Riccio 2*; *L. kirschneri* serovar *grippotyphosa*, type *Moskva V*; *L. biflexa* serovar *patoc,* type *Patoc*), and unrelated bacteria (*Streptococcus* spp.; *Eschericia coli*; *Klebsiella pneumoniae*; *Pseudomonas aeruginosa*; *Staphylococcus intermedius*; *Proteus vulgaris*; *Enterococcus faecalis*), as well as DNA from bovine and caprine faeces and clinical specimens from animals with previously diagnosed *Leptospira* infection, were used to test the specificity of the protocol.

A total of 25 μL of reaction mixture contained $10 \times$ buffer, 3 mM MgCl$_2$, 200 μM each deoxyribonucleotide triphosphate, 1 μM each primer (Sigma-Genosys), 0.5 U Taq DNA polymerase (Microtech, Italy). A fixed volume of DNA was used, 5 μL for the first cycle and 1 μL for the nested protocol. The cycling conditions were as follows: 95°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds for *Leptospira* and 59°C for 30 seconds for *C. burnetii*, and 72°C for 45 seconds, and 72°C for 10 minutes. In each set of reactions, a positive control (*L. interrogans* serovar *Bratislava,* type *Riccio 2*; *C. burnetii* 9 mile/I/EP1, ATCC VR-615), and a negative control (negative sample), as well as a negative reaction mix control (containing the reagents and water instead of DNA), were included in each run.

A previously published PCR protocol targeting the LipL32 gene of pathogenic *Leptospira* spp. was planned to confirm any case of positivity for *Leptospira* DNA.[24]

Results

A total of 151 specimens were examined in this study, originating from 103 cases in dogs (n = 94) and cats (n = 9), among which there were 5 abortions (4.8%), 3 stillbirths (2.9%), 24 cases of early neonatal mortality (23.3%), 18 cases of late neonatal mortality (17.5%), and 53 cases in which no age was recorded (51.4%).

All the samples tested were negative for *Leptospira* and *C. burnetii* DNAs. Detection limits of the *Leptospira* spp. PCR assay were 0.05 ng/μL in the first cycle and 0.5 pg/μL in the nested cycle. The nested protocol increased the sensitivity of the test 100 times. Considering that the genome of a single *Leptospira*, corresponding to 4.3×10^6 bp in *L. interrogans* and 3.6×10^6 bp in *L. biflexa*, weights 5-10 fg, the protocol was able to detect 50-100 bacteria/μL. All the tested *Leptospira* strains used as controls were positive, whereas no amplification was obtained from bacteria other than *Leptospira* spp. or bovine and caprine faeces, confirming the specificity of the test.

Although not systematically investigated, in 49 cases (47.6%) other likely causes of abortion and neonatal mortality were identified. Overall, CHV-1 was detected in 7.8% cases (6/77), and one of these showed a simultaneous coinfection with a beta-haemolytic strain of *E. coli*. FHV-1 was detected in 11% (1/9) and FPV in 9% (1/11) of the cases. Bacteriological examination was performed in 62 cases, 38 (61.3%) of which were positive. In particular, *E. coli* (20/38, 52.6%), *Staphylococcus* spp. (19/38, 50%), *Pseudomonas* spp. (8/38, 21%), *Proteus* spp. (8/38, 21%), *Klebsiella* spp. (8/38, 21%), *Streptococcus* spp. (1/38, 2.6%), *Enterobacter* spp. (1/38, 2.6%), and *Enterococcus* spp. (1/38, 2.6%) were isolated.

Discussion

The role of *C. burnettii* and *Leptospira* spp. in canine and feline abortion, stillbirth, and neonatal mortality is generally minimally investigated. In this study, no evidence of *C. burnetii* and *Leptospira* spp. DNA was found in cases of canine and feline abortion, stillbirth, and neonatal mortality submitted for the investigation of infectious causes; in fewer than the half of the cases had an infectious agent already been identified as a possible cause. The presence of *C. burnetii* and *Leptospira* has been previously reported in the same geographical area and time frame in which this study was conducted, although in different animal species; leptospiral DNA was found in equine abortion and stillbirth,[25] whereas *C. burnetii* has been found in sheep in the same area.[26] Moreover, diagnoses of cases of leptospirosis in dogs are reported regularly.[27] According to these previous studies, it might be supposed that our negative results could be related to the fact that cases in dogs and cats not exposed to risk factors for *Leptospira* spp. and *C. burnetii* infections were analysed in this study. Indeed, Q fever and coxiellosis are traditionally associated with contact with cattle, sheep, and goats, which are considered the main reservoir of infection. In a previous study, which tested canine sera to detect antibodies for *C. burnetii,* dogs that had contact with ruminants were 10 times more likely to be positive.[28] On the other hand, it has been observed that dogs living in kennels had a higher prevalence of *Leptospira* spp. when compared with client-owned dogs, and that, despite vaccination, risk factors for exposure are relevant for the infection.[29] Similarly, in cats, a significantly higher *C.burnetii* antibody positivity rate was found in stray cats than in pets,[30] and leptospiral antibody prevalence is higher in outdoor cats, as well as in cats living in urban areas and cats that are known hunters.[31] It has been suggested that the active predatory behaviour of some cats increases the chance of infection for both *C. burnetii* and *Leptospira* spp., due to contact with reservoirs such as rodents.[7,16] Unfortunately, in the current study, a serological investigation to establish the exposure to these pathogens was not performed in the bitches and queens, and no indication of breed or lifestyle was specifically requested upon admission of the specimens. Further studies targeting especially populations at risk for these infections should be designed, using animals exposed to water sources or environments contaminated by faeces, urine, or parturition products of animal reservoirs in order to increase the likelihood of finding positive results and determining whether *C. burnetii* and *Leptospira* spp. are responsible for canine and feline abortion and neonatal losses.

Overall, it is possible that only a specific subpopulation of dogs and cats, especially pedigree cats and pets, was submitted for diagnostic examination in cases of abortion and neonatal mortality in the current study. A previous retrospective analysis of feline neonatal mortality in the United Kingdom suggested that pedigree cat breeders generally investigate the reasons for such deaths[2] and it is possible that infectious diseases associated with environmental exposure are underrepresented in this group of animals.

The failure to confirm the presence of *C. burnetii* and *Leptospira* spp. in this study could also be attributed to the timing of sample collection and to the nonoptimal storage conditions that can occur, according to the specific infection.[32] Additionally, in this study placentas were very rarely submitted for analysis because bitches and queens commonly ingest them. Examination of the placenta and umbilical cord is recognized as an important means of diagnosing leptospirosis in some species.[33] In addition, there is strong evidence that the placenta represents the critical tissue also for the detection of *C. burnetii*, because up to 10^9 *C. burnetii* per g of placental tissue are released in some species at the time of delivery.[34]

These results showed that *C. burnetii* and *Leptospira* spp. are probably not common agents of canine abortion or neonatal deaths, whereas a higher number of feline cases is needed to arrive at conclusions. However, considering that reproductive failure can occur following *C. burnetii* and *Leptospira* spp. infection, these 2 agents should always be considered in the differential diagnosis in any case of abortion, stillbirth, and neonatal mortality, especially when none of the usually suspected pathogens causing reproductive abnormalities has been identified.[12,35] Moreover, due to the zoonotic potential of these aetiological agents and the close relationship between companion animals and humans, an active surveillance programme encompassing a larger number of canine and feline abortions/stillbirths/neonatal deaths, including a standardized form for recording signals and investigating some risk factors, could be useful to identify these uncommon agents of abortion and neonatal losses and improve the epidemiological knowledge of them.

Acknowledgments

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