



Published in final edited form as:

J Wildl Dis. 2022 April 01; 58(2): 333–340. doi:10.7589/JWD-D-21-00037.

HISTOLOGIC LESIONS IN PLACENTAS OF NORTHERN FUR SEALS (*CALLORHINUS URSINUS*) FROM A POPULATION WITH HIGH PLACENTAL PREVALENCE OF *COXIELLA BURNETII*

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Abstract

Coxiella burnetii is an intracellular bacterial pathogen that can be associated with significant reproductive disease or acute mortality in livestock and wildlife. A novel marine mammal-associated strain of *C. burnetii* has been identified in pinnipeds of the northwestern Pacific Ocean. Little is known about *C. burnetii* infection in regard to reproductive success or population status. Our objective was to characterize the severity and extent of histologic lesions in 117 opportunistically collected placentas from presumed-normal northern fur seals (*Callorhinus ursinus*) in July 2011 on St. Paul Island, Alaska, US, where a high placental prevalence of *C. burnetii* had been reported. Sections were examined by histology and immunohistochemistry and impression smears with modified acid-fast stain. The nature and frequency of histologic changes were compared with target *COM1* PCR-confirmed *C. burnetii* positive and negative placentas. Overall, histologic changes were similar to placental lesions described in aborting ruminants; however, changes were variable within and between placentas. Vasculitis and occasional intracellular bacteria were seen only in *C. burnetii* PCR-positive placentas. Dystrophic mineralization, edema, and inflammation were seen in PCR-positive and negative placentas, although they were statistically more common in PCR-positive placentas. Results suggest that *C. burnetii* and associated pathologic changes are multifocal and variable in placentas from these

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presumably live-born pups. Therefore, multiple sections of tissue from different placental areas should be examined microscopically, and screened by PCR, to ensure accurate diagnosis as the genomes per gram of placenta may not necessarily represent the severity of placental disease. These limitations should inform field biologists, diagnosticians, and pathologists how best to screen and sample for pathogens and histopathology in marine mammal placental samples.

Keywords

Callorhinus ursinus; *Coxiella burnetii*; histopathology; northern fur seal; placenta

INTRODUCTION

The decline in recent decades of the northern fur seal (*Callorhinus ursinus*) population on St. Paul Island, Alaska, US (Towell et al. 2006) has prompted investigations into the cause (Spraker and Lander 2010). *Coxiella burnetii* has been detected by PCR in approximately 75% of northern fur seal placentas collected from live births on St. Paul Island since 2010 (Duncan et al. 2012, 2013). This pathogen infects most, if not all, species of animals (McQuiston and Childs 2002), and high titers of *C. burnetii* antibodies have been identified in sympatric species including the endangered Steller sea lion (*Eumetopias jubatus*; Minor et al. 2013). Infections with *C. burnetii* have been sporadically reported in free-ranging, stranded marine mammals in California and Washington State, US, and the impact of this pathogen to individual and population health is unknown (Kersh et al. 2010, 2012). The bacterial strain reported in marine mammals has not been identified in terrestrial mammals to date, and there is molecular evidence that this strain diverges significantly from other *C. burnetii* genotypes (Kersh et al. 2010). This suggests marine mammal-specific adaptation of this *C. burnetii* strain and raises the possibility that disease manifestations may be distinct in these species. In domestic ruminants, the bacterium causes reproductive failure in sheep and goats and, with introduction of an asymptomatic carrier, can cause abortion storms, whereas in dairy cattle, asymptomatic infection, persistent shedding in milk, and sporadic abortions have been reported (Maurin and Raoult 1999). There is little information about the clinical significance of infection in other domestic and wildlife species (Agerholm 2013). Given these known reproductive effects, identification of this pathogen in northern fur seals with a protracted history of declining population numbers prompted this investigation into the potential role of *C. burnetii* in fetal loss and lack of population recruitment in this region.

Placental integrity is a critical component of fetal development. In humans, placental pathology is well correlated to both short- and long-term health outcomes for mother and offspring (Redline 2008). With this in mind, routine examination of postparturient placenta can give insight into individual and population health. Unfortunately, in animals far less is known about sublethal placental disease, particularly in wildlife species. This is probably because we rarely have the opportunity to look at them in a systematic way. As a result, considerably less is known about subclinical placental disease in animals, particularly wildlife species. In marine mammals, descriptions of placental lesions are primarily from a small number of opportunistically collected tissues when field personnel and resources are available to investigate unusual morbidity or mortality events. Histopathologic lesions

attributed to *C. burnetii* infection have been described in Steller sea lions, Pacific harbor seals (*Phoca vitulina richardsi*), and northern fur seals and include variable inflammation, necrosis, and typically a myriad of intracytoplasmic bacteria (Lapointe et al. 1999; Kersh et al. 2010, 2012). At present, there is little epidemiologic, clinical, and pathologic information on where pinnipeds and otariids lie on the spectrum of clinical consequences of infection in comparison to goats, sheep, and dairy cattle. This study was undertaken to enhance our understanding of placental pathology, diagnostic tools, and potential implications of *C. burnetii* infection within a declining population as well as to aid in future investigations.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue from 117 placentas collected as previously reported (Duncan et al. 2012) was used in this study. These placentas were collected in July 2011 from a single northern fur seal rookery on St. Paul Island, Alaska. At the field laboratory each placenta was grossly evaluated by a veterinary pathologist (C.D.), labeled, and the sample date recorded. Although subsampling was opportunistic, a baseline of six, 2–3 cm² samples were taken from different sites around the labyrinth, with some at the junction of the labyrinth and the marginal hematoma. These correlated to roughly one sample from each side of the midline on the proximal, middle, and distal third intervals along the length of the placenta. Samples were prepared for histologic examination as described in Duncan et al. (2012). A total of 757 sample sections were prepared and reviewed, and samples mentioned hereafter refer to these sections. A 2-g fresh sample from each placenta for PCR was obtained adjacent to one of the six fixed tissue sections and frozen at –80 C in a U-line Whirl-Pak bag (Whirl-Pak, Madison, Wisconsin, USA).

From 30 placentas, direct impression smears were prepared from six locations adjacent to those sampled for histopathology, air dried, and stained with a modified acid-fast stain (Bildfell et al. 2000). A *C. burnetii* *COM1* (target gene for *C. burnetii*) PCR analysis was performed according to Kersh et al. (2010) using an Applied Biosystems 7500 FAST PCR machine (Waltham, Massachusetts, USA) and as reported in Duncan et al. (2012). The estimate of genomes per gram provided by *COM1* PCR is considered to be more accurate than using the IS1111 PCR target due to variation in IS1111 copy number and inefficient recognition of the IS1111 target in marine mammal-associated *Coxiella* (Kersh et al. 2010). These placentas were also screened for *Brucella* spp. (reported in Duncan et al. 2014).

For this study, all 757 histologic sections of each placenta were reviewed by two pathologists (C.D., C.F.). A subset of slides from both infected and noninfected placentas with a range of histologic lesions were examined by two other pathologists (R.A.F., S.R.). Following the initial histologic review, additional tissue sections were prepared if a component of the placenta, usually the marginal hematoma/hemophagocytic area, was not present in the initial sections. Each section was reviewed systematically, and findings were noted as presence/absence of changes as well as described with free text. For each placenta, both fetal and maternal components, the placental labyrinth, the stroma, and the hemophagocytic regions were evaluated and scored independently. Each sample was reviewed for increased infiltration of leukocytes (inflammation), necrosis, edema, and bacteria. If present, mineralization and vasculitis were also noted. Immunohistochemistry

(IHC) for *C. burnetii* was performed as described in Duncan et al. (2012) on a single sample from each of the 117 placentas. For each placenta the sample with the most severe histologic lesions was selected for IHC.

Descriptive and comparative statistics were performed using commercially available software (SPSS version 25, IBM, Armonk, New York, USA). Associations between PCR status (positive or negative) and the presence of particular histologic changes were evaluated using Pearson chi-square test and odds ratios (OR) with 95% confidence intervals (CI) or Fisher's exact tests when indicated by observed or expected frequency counts. Histopathology observed in different sections of a single placenta were later aggregated into a summary score for each placenta that included both dichotomous outcomes (presence or absence of histologic change in any sections) and the percentage of sections in that placenta in which the change was observed. To determine if the frequency of lesions differed between *C. burnetii* PCR-positive and negative placentas, both a chi-square (categorical data) and analysis of variance (continuous data) were performed.

RESULTS

None of the 117 placentas examined had gross lesions. The number of placentas, tissue sections, and testing results are summarized in Table 1. Of the fresh samples taken from each placenta, 89 (76%) were positive for *C. burnetii* by *COM1* PCR. The *COM1* genomes per gram of tissue ranged from 312 to 270,929,000 with a mean and SD of $4,288,054 \pm 29,199,616$, respectively. Twenty-eight placentas (24%) were negative for *C. burnetii*.

Histologically, intracytoplasmic bacteria were observed in 7/757 (0.9%) sections from a total of 5/117 (4%) placentas (Fig. 1A, C, D). The organisms were localized in the hemophagocytic zone in four sections and in the placental labyrinth in three of the sections. Two samples had organisms in both the hemophagocytic zone and the placental labyrinth. Bacteria were noted along the placental surface in a single section which also had abundant organisms in the labyrinth in one section and in the hemophagocytic zone in another sample with a very high number (15,229,000) of genomes per gram of tissue. Histopathology revealed inflammation and necrosis associated with the bacteria ranging from rare foci to very severe, regionally extensive bands of neutrophils. Immunohistochemistry confirmed intralesional *C. burnetii*. It is important to note that IHC did not reveal bacteria in any *C. burnetii* PCR-positive placentas in which no bacteria were visualized on histology.

Lymphoplasmacytic vasculitis (Fig. 1B) was present in 2/7 sections with intracytoplasmic bacteria and in seven additional PCR-positive placentas with no discernible microorganisms. All placentas with vasculitis were positive for *C. burnetii* by PCR, and a *Brucella* sp. was identified in a single placenta by IHC and PCR (Duncan et al. 2014). Although often not in the plane of section with the vasculitis, all placentas with inflammation of the vessels had areas of necrosis, often wedge shaped and radiating from the fetal to maternal surface, consistent with infarction. In 1/9 sections with vasculitis, a large thrombus was present in the affected vessel.

Dystrophic mineralization was often present (Fig. 1A) and statistically more common in sections from PCR-positive placentas (82%) compared to PCR-negative (67%) placentas (OR 2.3, 95% CI 1.6–3.3). Foci of mineralization were restricted to the labyrinth, often oriented around large blood vessels but also randomly distributed throughout the interstitium. Subjectively, the quantity of mineral within a section appeared greater in areas with more-severe lesions; there was a statistically significant association between the presence of mineral and necrosis (OR 1.7, 95% CI 1.1–2.5) but not of inflammation and mineral deposition (OR 1.4, 95% CI 0.9–2.1) within a section.

Inflammation was seen in 31% of sections of placenta from PCR-positive placentas, but in only 18% of sections from PCR-negative placentas (OR 2.1, 95% CI 1.4–3.1). Both inflammation and coagulative necrosis (Fig. 1C) were statistically more common in the labyrinth of sections from PCR-positive placentas compared to PCR-negative placentas. Necrosis was identified in 33% of sections from PCR-positive placentas compared to 21% of sections from PCR-negative placentas (OR 1.8, 95% CI 1.23–2.6). Areas of inflammation were most often identified just below the fetal surface or at the base of the placental labyrinth and were usually associated with linear or wedge-shaped necrosis. The severity of inflammation was extremely variable between sections for an individual placenta. In most cases inflammation of the labyrinth was neutrophilic with peripheral aggregates of lymphocytes and plasma cells. Rarely there was a prominent eosinophilic ($n=5$) or histiocytic ($n=4$) infiltrate. Microfilaria were seen in the placental labyrinth in one section. Although parasite speciation was not conducted, these nematodes were histologically consistent with *Acanthocheilonema odendhali* (Kuzmina et al. 2013).

Hemophagocytic regions were present in 105 sections from 82 placentas. While there was no statistically significant difference in the frequency of histopathologic features between PCR-positive and PCR-negative placentas ($P>0.05$), inflammation and necrosis were more common in hemophagocytic areas relative to the remainder of the placental labyrinth. Inflammation was identified in 74% of the sections and necrosis in 88% of sections. Often inflammation and necrosis were prominent in these areas and perilabyrinthic tissues were unaffected. Inflammation was predominantly neutrophilic as in the remainder of the labyrinth.

The maternal surface of all placentas had a variable amount of environmental and cellular debris, fibrin, and acute hemorrhage. The frequency of inflammation and necrosis did not differ significantly between PCR-positive and PCR-negative placentas ($P>0.05$ for both). Impression smears were prepared and evaluated for the six histology sampling sites from 30 placentas for a total of 180 impressions. No bacteria were observed by cytology, although 15 (50%) of the placentas were *C. burnetii*-positive by PCR.

In 78% of the examined sections, supporting stroma had frequent perivascular patchy areas of edema with occasional lymphocytes and plasma cells, which was statistically more common in sections from PCR-positive compared to PCR-negative placentas (edema OR 3.2, 95% CI 2.2–4.5; inflammation OR 2.8, 95% CI 2.0–4.1). Edematous regions were primarily adjacent to large blood vessels. In general, stromal changes did not appear to correlate with the severity of lesions in the placental labyrinth.

When individual sections were aggregated to create a categoric (presence/absence) summary score for each placenta, the presence of labyrinth inflammation and intralesional *C. burnetii* organisms was statistically increased in PCR-positive placentas ($P=0.008$) but not the presence of mineral, surface inflammation, surface necrosis, labyrinth necrosis, stromal inflammation, stromal edema, vasculitis, mineralization, hemophagocytic zone inflammation, or hemophagocytic zone necrosis ($P>0.05$ for all). When individual sections were aggregated to create a continuous summary score reflecting the percentage of sections from each case that possessed the histologic attribute in question, there was statistically more labyrinth necrosis ($P=0.024$) and vasculitis ($P=0.002$) in PCR-positive placentas, but significantly less necrosis in hemophagocytic zones in PCR-positive placentas relative to PCR-negative ones ($P<0.001$).

DISCUSSION

Our findings may be used to facilitate sampling and testing recommendations for pinniped placentas in areas with a clinical suspicion of *C. burnetii* exposure. Given the multifocal nature of infection and lesions, histologic sections should be collected from multiple locations of the placenta including both the labyrinth and marginal hematoma/hemophagocytic areas. A single section of fresh tissue appears sufficiently sensitive to detect the organism by PCR; however, our findings suggest that the genomes per gram of tissue in the tested sample may not reflect the overall severity of lesions or load of bacteria in the whole tissue: severe inflammation, necrosis, and vasculitis were seen histologically in placentas where the genomes per gram were as low as 1,780 while, paradoxically, some histologic sections from placentas with extremely high genomes per gram ($>15,000,000$) of tissue were devoid of histologic lesions.

While pinniped placentas are most structurally similar to dogs and cats, there is little published on *C. burnetii* lesions in pinnipeds. When comparing histologic lesions identified in *C. burnetii* PCR-positive versus negative northern fur seal placentas, the statistically significant microscopic features are consistent with those previously described in ruminants with experimental and naturally occurring *C. burnetii* exposures, but with some notable differences. When present in placental sections, intracytoplasmic bacteria were readily identified in northern fur seal placentas, and IHC did not identify bacteria not initially visualized with routine histology. This was in contrast to cattle, where intracytoplasmic organisms were apparently harder to identify on histologic sections relative to sheep (van Moll et al. 1993), and IHC helped to identify *C. burnetii* as the etiologic agent in a subset (30%) of cases where the organism was not identified on initial histologic examination using H&E or Macchiavello stains (Bildfell et al. 2000). Previous findings in harbor seal placentas also noted a paucity of IHC bacterial detection; this discrepancy was attributed to the focal nature of infection and relatively low bacterial burden in many of the samples (Kersh et al. 2012).

In the absence of identifiable bacteria within the tissue, detection of vasculitis and abundant inflammation are indicators of *C. burnetii* infection in northern fur seals. All placentas with vasculitis were *C. burnetii* positive, although one of these was also positive for *Brucella* spp. by PCR and IHC (Duncan et al. 2014). When vasculitis was identified, inflammation and

necrosis were a prominent feature in at least one piece of tissue from that placenta. These are similar lesions to those seen in sheep, goats, and cattle where *C. burnetii* is known to cause abortion (Zeman et al. 1989; Moore et al. 1991; van Moll et al. 1993; Oporto et al. 2006; Sánchez et al. 2006). Mineralization, stromal inflammation, and stromal edema were more commonly identified in *C. burnetii* PCR-positive placentas; however, the strength of these associations was relatively weak, suggesting these changes may not be specific to *C. burnetii* infection. Therefore, these features alone are insufficient to suggest *C. burnetii* infection. Also, these histologic changes in many sections were mild, even if tissue from other locations in the same placenta were severely affected.

The pathogenesis of severe inflammation, necrosis, and more-numerous bacteria within hemophagocytic areas are uncertain but consistent with the distribution of other intracellular abortive bacteria in similar placentas such as dogs with *Brucella canis* infection (Foster 2016). In goats, areas of hemorrhage from maternal vessels beginning on day 60 of pregnancy have been proposed to be a good location for contact between maternal blood and placental trophoblasts, facilitating infection of these cells by blood-borne pathogens such as *C. burnetii* (Sánchez et al. 2006). Because the hemophagocytic area of pinnipeds has been proposed as an important site for uptake of iron for the developing fetus (Rowlands 1966), it is also possible that trophoblasts in these areas sequester cofactors that facilitate propagation of intracellular pathogens. However, the iron requirements for *C. burnetii* may be lower than those of other pathogens (Briggs et al. 2008).

Placental impression smears have been suggested to be a good screening tool for *C. burnetii* infection in cattle, correctly identifying 9/10 of IHC-positive bovine placentas (Bildfell et al. 2000), and abundant cellular debris, inflammatory cells, and extracellular organisms have been described on the surface of infected goat placentas (Zeman et al. 1989; Sánchez et al. 2006). In a single harbor seal placenta, occasional exfoliated cells contained cytoplasmic aggregates of bacteria (Lapointe et al. 1999), suggesting that organisms may also be identified cytologically in these species. However, in northern fur seals impression smears may not be a sensitive way to identify *C. burnetii* infection. In contrast to other species, superficial bacteria were only identified histologically in a single *C. burnetii*-positive northern fur seal placenta, and none of the placental impression smears reviewed cytologically had identifiable bacteria in this case series.

Histopathology of *C. burnetii*-infected northern fur seal placentas reveals that some lesions are comparable in composition and severity to those described for abortion in domestic animals. All placentas included in the histologic review were collected from the rookery without an apparent or associated dead pup; as such they were assumed to have come from live, full-term births. At present it is not possible to deduce the impact of the observed placental changes on placental integrity or the developing fetus that may influence in utero or postpartum survival. In order to better determine the significance of infection for the developing fetus, a mechanism for assessing the overall percentage of compromised tissue, and correlation to fetal outcome, is needed in addition to accounting for additional factors that could influence placental integrity.

A longitudinal study of mortality in northern fur seal neonates demonstrated an unusually high rate of perinatal mortality that has been increasing over time; while some of these deaths are explained by dystocia and trauma, it has also been hypothesized that weak or unthrifty pups may fail to nurse and die of starvation (Spraker and Lander 2010). Placental insufficiency is a well-known cause of intrauterine growth restriction in humans; however, less is known about this phenomenon in domestic or wild animals. *Coxiella burnetii* is known to alter apoptosis in northern fur seal placentas (Myers et al. 2013), and occasionally infect the fetus in utero (C.D. pers. comm.), suggesting that it may have some impact on fetal development. Given that birth weight is an important factor influencing survival and fitness in northern fur seal (Calambokidis and Gentry 1985; Baker and Fowler 1992), and the northern fur seal population of the Pribilof Islands has experienced a significant population decline, a better understanding of factors that influence in utero and postpartum survival is critical.

ACKNOWLEDGMENTS

This project was supported by the John H. Prescott Marine Mammal Rescue Assistance Grant Program. Authors would also like to acknowledge the support of Cody Minor for tissue processing and Tylor Zumbusch for assistance with cytology. All samples were collected under authorization of US Marine Mammal Permit 782-708 issued to the National Marine Mammal Laboratory, Alaska Fisheries Science Center, National Oceanic and Atmospheric Administration (NOAA) Fisheries, Seattle, WA 98115. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control (CDC) or the Department of Health and Human Services.

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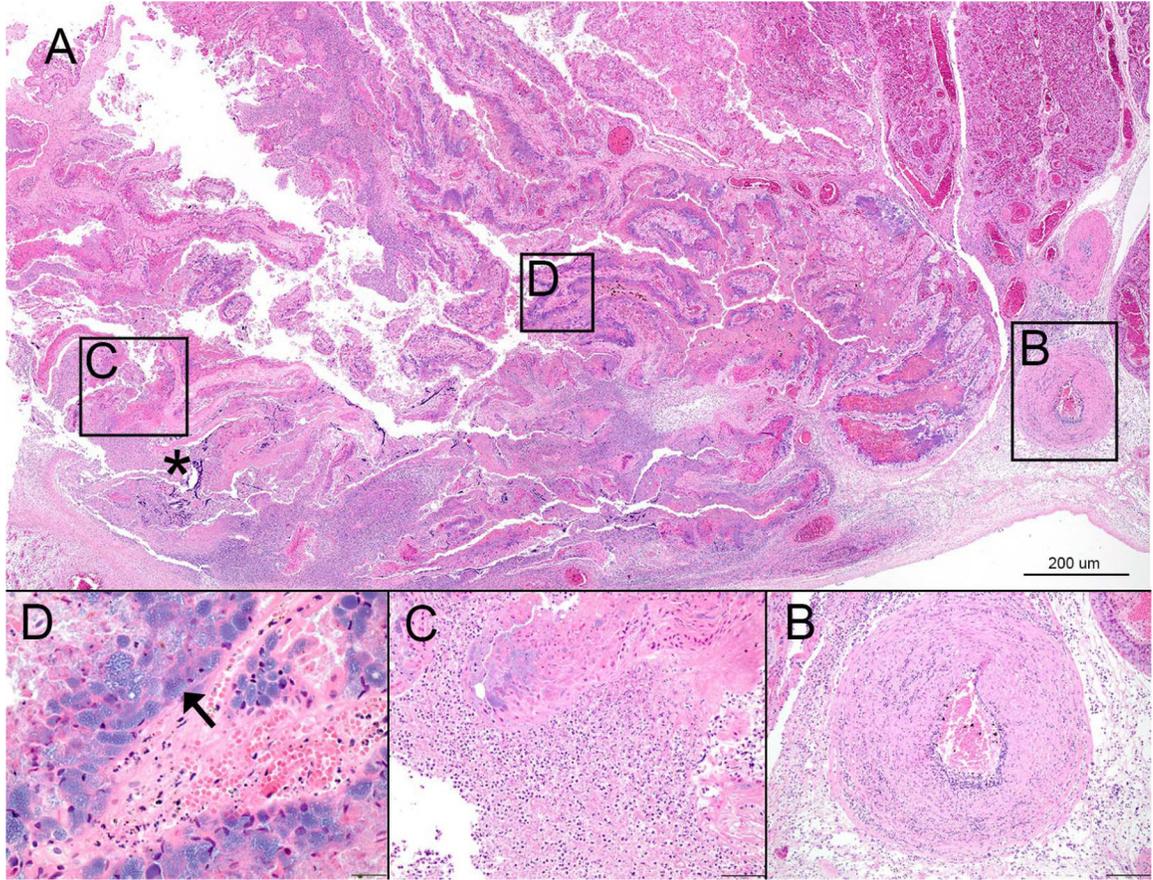


Figure 1.
A) Photomicrograph (4×) of placenta from a *Coxiella burnetii*-infected northern fur seal (*Callorhinus ursinus*) collected in July 2011 on St. Paul Island Alaska, USA, with severe placental lesions including: B) vasculitis with stromal edema and inflammation (10×); C) regionally extensive areas of necrosis and suppurative inflammation (10×); with D) numerous trophoblasts containing intracytoplasmic bacteria (arrow; 40×). Foci of mineralization are present adjacent to the area of necrosis and inflammation (*). H&E.

Table 1.

The number of northern fur seal (*Callorhinus ursinus*) placentas collected in July 2011 on St. Paul Island, Alaska, USA, and the number of individual samples tested and found positive for *Coxiella burnetii* by *COM1*-target PCR, cytology, histopathology, and immunohistochemistry respectively.

Test	Placentas, n	Samples, n	Positive placentas (%)	Positive samples (%)
<i>COM1</i> -PCR	117	117	89 (76)	89 (76)
Cytology	30	180	0 (0)	0 (0)
Histopathology	117	757	5 (4) with visible bacteria	7 (0.9) with visible bacteria
Immunohistochemistry	117	117	5 (4) with visible bacteria	Not applicable

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