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Campylobacter fetus in Abomasal Fluid from Spontaneously Aborted Bovine and Ovine Fetuses

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ABSTRACT

***Background*:** Pregnancy losses are a major concern in livestock industry due to their economic impact on producers. *Campylobacter fetus* subspecies *fetus* (Cff) and *C. fetus* subspecies *venerealis* (Cfv) are directly related to reproductive failures in ruminants. Cff colonizes the gastrointestinal tract of a wide range of hosts leading to abortion, while Cfv is restricted to genital tract being generally associated to infertility in bovine. Considering the great economic losses related to campylobacteriosis in cattle and ovine herds, this study aims to investigate the occurrence of *C. fetus*, considering Cff and Cfv subspecies, in bovine and ovine spontaneously aborted fetuses in state of Rio Grande do Sul, Brazil.
***Materials, Methods & Results*:** In this study, samples of abomasal fluid collected from 30 spontaneously aborted bovine (n = 18) and ovine (n = 12) fetuses were investigated for the detection of *Campylobacter fetus* throughout conventional PCR. Positive fetuses for *C. fetus* presence were further analyzed by molecular assays for Cff and Cfv detection, in order to determine subspecies identification. When available, samples of the main organs of the thoracic and abdominal cavities, as well as the brain, skeletal muscle, eyelid, skin, and placenta were collected for further histopathological analyses and bacterial culture, aiming to assess the presence of infection lesions and pathogens in those sites, respectively. Additionally, RT-qPCR assays were also performed for the detection of ruminant pestivirus, in order to detect bovine viral diarrhea cases. Throughout the present methodology, *C. fetus* was detected in the abomasal fluid samples of 2 bovine fetuses, being both identified as Cfv subspecies by PCR. Histopathological analyses demonstrated that macroscopic and microscopic changes found in the Cfv-positive animals were not either specific or directly related to *Campylobacter* infections. Moreover, no significant bacterial growth was observed in microbiological culture from the collected tissues, and both fetuses were negative for ruminant pestivirus. Differently, there was no detection of *C. fetus* in any of the analyzed ovine fetuses.
***Discussion*:** Considering that abortion diagnosis rates reported in cattle and sheep industry are highly variable among the published studies, and that abortion diagnoses are commonly inconclusive due to difficulties in sampling methodology and inadequate identification of the pathogen involved, it is important to investigate the etiological causes of abortion the herds for better understanding the causes of pregnancy issues and monitoring their occurrence. In addition, the absence of pathognomonic lesions in the tissues investigated in the histopathological analyses observed in this study strongly suggests that well-known etiological agents commonly associated to abortion, such as *Leptospira* spp., *Toxoplasma* spp., *Chlamydia* spp. and *Neospora caninum*, are unlikely to be the cause of infection of the analyzed fetuses. Taking this into account, the presence of *C. fetus* in the abomasal fluid samples from two bovine fetuses demonstrated in the present study suggests the possible association of Cfv not only with infertility, but also with cases of bovine abortion, highlighting the importance of investigating unusual causal agents of abortions in sheep and cattle. Overall, an adequate diagnosis is essential for establishing better prevention strategies to avoid the circulation of abortion-related infectious agents in the herds.

Keywords: campylobacteriosis, molecular diagnosis, venereal disease, abortion, reproductive disease.

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INTRODUCTION

Pregnancy losses are a major concern in livestock industry causing great economic losses. In cattle, reproductive issues are commonly associated to Bovine virus diarrhea (BVD), Brucellosis, Campylobacteriosis, Infectious Bovine Rhinotracheitis, Leptospirosis, Neosporosis, and Trichomoniasis [8,13]. In sheep, they are related to Chlamydial infection, Campylobacteriosis, Toxoplasmosis, Salmonellosis, Coxiellosis, Border Disease, and Cache Valley Disease [8].

Campylobacter fetus has 2 subspecies directly related to reproductive issues in ruminants: *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv) [23]. They show high genetic similarity, but different epidemiological behaviors. Cff affects a wide range of hosts by colonizing their gastrointestinal tract, commonly related to ovine and bovine abortion, while Cfv is restricted to bovine genital tract leading to infertility cases mainly [21].

In cattle, abortions are generally associated with Cff, but can also result from bovine genital campylobacteriosis (BGC) caused by Cfv. Males are asymptomatic carriers and transmission may occur during the breeding. BGC is spread worldwide, thus the high circulation of Cfv leads to infertility and sporadic abortion [10,16,19,20,27].

In sheep, there are few reports about abortion associated to *C. fetus* or the occurrence of Cff infection [6,9,12,22]. In Brazil, only one study has been published lately about the occurrence of Cff in ovine gastrointestinal tract [14].

The present study aims to investigate the occurrence of *C. fetus* in bovine and ovine spontaneously aborted fetuses in order to introduce data about the occurrence of these pathogens in abortion in South Brazil.

MATERIALS AND METHODS

Sample collection and processing

A total of 30 spontaneously aborted bovine (n = 18) and ovine (n = 12) fetuses received for *post mortem* examination from June 2019 to December 2020 were included in this study. Farm and herd information were obtained with referring veterinarians and farm owners. During the necropsy, estimated gestational age was measured through the measurement of crown-rump length and gross lesions were recorded, when present. Sample collection and further analyses were performed according to Figure 1.

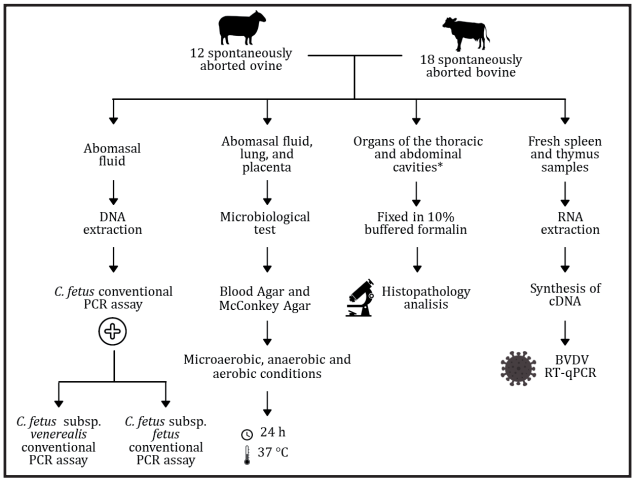

Figure 1. Workflow of the analyses: Abomasal fluid samples were collected from bovine and ovine fetuses for molecular diagnoses of *Campylobacter fetus*. Tissue samples were submitted to histopathological analyses, bacterial culture and RT-qPCR assays for BVDV detection.

Abomasum contents were collected with sterile syringes through puncture, being a fresh aliquot subjected to microbial culture in Blood Agar Base[1] supplemented with 5% of ovine blood and MacConkey Agar[1] and incubated in micro-aerobic, anaerobic and aerobic conditions at 37°C for 48 h. When available, fresh samples of lung and placenta were also cultivated.

Samples of the main organs of the thoracic and abdominal cavities, as well as the brain, skeletal muscle, eyelid, skin, and placenta, when available, were collected, fixed in 10% buffered formalin, routinely processed for histopathology and stained with hematoxylin[2] and eosin[3]. Tissue sections were observed under light microscopy and histopathological lesions were recorded. Additionally, fresh spleen and thymus samples were collected for further RNA isolation for ruminant pestivirus detection.

DNA isolation from abomasum content and Campylobacter fetus *subsp.* fetus *and* Campylobacter fetus *subsp.* venerealis *detection by molecular analyses*

Aliquots of 1 mL of abomasum content were used for DNA isolation. Samples were previously treated with 0.1V of dithiothreitol (DTT) 0.2% and incubated at 37°C for 20min, in order to decrease viscosity. Then, samples were homogenized in vortex and cells were harvested by centrifugation at 11,000 x *g* for 5min. DNA extraction was performed following of BIOPUR Mini Spin Plus Extraction Kit protocol 1[4], including 10µL of RNAse A (10 ng/µL) as indicated by

the manufacturer instructions. Total DNA was eluted in 30 μL of elution buffer.

Conventional PCR assays were performed in order to detect *C. fetus* (Table 1). Positive samples were further tested for subspecies identification, using primer sets for Cff and Cfv (Table 1). PCR assays were performed in 12 μL reactions, using 1 U Taq DNA polymerase[5], Taq DNA Buffer 10x, 50 mM of $MgCl_2$, 1 mM of dNTP, 10 pmol of each primer, and 50 ng of DNA. Amplification conditions comprised: an initial denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 20 s, melting temperature as indicated in Table 1 for 20 s, and 72°C for 2 min; and a final extension step at 72°C for 10 min. Strains Cff ATCC 27374 and Cfv ATCC 19438 were used as positive controls.

RNA isolation from abomasum content and ruminant pestivirus detection by molecular analyses

Total RNA was isolated using Quick-Zol[6] according to the manufacturer's instructions. cDNA synthesis and PCR were performed using a GoScript™ Reverse Transcription System and GoTaq G2 Hot Start Polymerase[7]. The PCR amplification resulted in a product of 118 bp of 5`UTR of ruminant pestivirus that include Bovine Viral Diarrhea Virus-1 (BVDV-1), BVDV-2, Border Disease Virus (BDV), and HoBi-like pestivirus [26].

Table 1. PCR primers and targets for *Campylobacter fetus, Campylobacter fetus* subsp. *fetus* (Cff) and *Campylobacter fetus* subsp. *venereali*s (Cfv).

Campylobacter target	Code	Target gene	Primer sequence (5' to 3')	Product length (bp)	Tm* (ºC)	Reference
C. fetus	CAMPG-F	nahE	GGTTATTTTTTATAACTGTAGGAATGCAGAT	390	54	[1]
	CAMPG-R		GATCGCTTAAATCTTGTACTTTTAGCTTTT			
*Cff***	MG3F	cstA	GGTAGCCGCAGCTGCTAAGAT	960	52	[11]
	MG4R		TAGCTACAATAACGACAACT			
*Cfv****	CVEN-F	ISCfe1	GGTGGAGAGCGTAGATATAAATTAG	455	52	[5,24]
	CVEN-R		CCATAAAGCCTAGCTGAAAAAACTG			

*Melting temperature (Tm). ***Campylobacter fetus* subsp. *Fetus.* ****Campylobacter fetus* subsp. *venerealis.*

RESULTS

A total of 30 spontaneously aborted bovine (18) and ovine fetuses (12) were analyzed in the current study, in which *Campylobacter fetus* was detected by molecular analyses in 2 (6.6%) abomasal fluid samples. *C. fetus* was identified only in 2 bovine fetuses, named F1 and F2 (Table 2). The samples from fetuses F1 and F2, which were positive to the *C. fetus* PCR reactions, were then subjected to PCR assays for Cff and Cfv detection, in order to identify which subspecies was involved in the abortion. After that, we detected Cfv in both fetuses by subspecies PCR assays (Table 3).

Both fetuses were third-trimester male calves (7 months of gestation), referred from farms located in the state of Rio Grande do Sul, South Brazil (Table 3). The farm of fetus F1 was an extensive cow-calf operation, with 600 breeding cows. Reproductive management used to be conducted with fixed-time artificial insemination, and bulls were used to breed empty cows only. The farm owner reported 10% of embryonic losses from 30 to 60 days of gestation and several third-trimester abortions between 2018 and 2019. The farm of fetus F2 was a dairy operation with 170 cows housed in a free-stall barn. All cows were artificially inseminated, and nine abortions were recorded in 2019.

In farms of fetuses F1 and F2, vaccination was conducted against Bovine Infectious Rhinotracheitis, Bovine Viral Diarrhea Virus (BVDV), Parainfluenza type 3 (PI3), Bovine Respiratory Syncytial Virus (BRSV), and *Leptospira interrogans* serovars Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona. No vaccination against *C. fetus* used to be conducted in both farms.

At *post mortem* examination, fetuses F1 and F2 were moderately and mildly autolyzed, respectively. None of the samples from both fetuses submitted for microbiological culture presented significant bacterial growth. In addition, both fetuses had negative PCR results for ruminant pestivirus (Table 3). The macroscopic and microscopic changes found in the Cfv-positive animals (F1 and F2) were not

either specific or directly related to *Campylobacter* infections. In F1 was observed moderate deposition of friable yellowish material in cotyledons, discrete multifocal necrosis of the chorionic epithelium of the placenta associated with deposition of cell debris and discrete inflammatory infiltrate of lymphocytes and macrophages, discrete multifocal inflammatory infiltrate of lymphocytes and macrophages in alveolar spaces of the lungs and moderate accumulation of intra-alveolar golden granular pigment (meconium), and: marked diffuse autolysis in liver and spleen. No alteration was observed in F2.

Table 2. PCR results for *Campylobacter fetus*, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* from abomasal fluid from aborted bovine and ovine fetuses.

Animal	Positive for *C. fetus*	Negative for *C. fetus*	Positive for *C. fetus* subsp. *fetus*	Positive for *C. fetus* subsp. *venerealis*
Cattle	2	16	0	2
Sheep	0	12	0	0
Total	2	28	0	2

Table 3. Overview information of the *Campylobacter fetus* subsp. *venerealis*-positive fetuses.

Fetus ID	Specie	Gender	Breed	Gestational age	RT-PCR BVDV	Microbiological test
F1	Bovine	Male	Angus	7 months	ND	No growth
F2	Bovine	Male	Holstain	7 months	ND	No growth

Gestational age was an estimative. ND: indicates pestivirus was not detected in the analysed sample by RT-PCR.

DISCUSSION

Diagnostic rates of abortion in cattle and sheep reported by different studies are highly variable. Complementary tests significantly impact on greater diagnosis proportion [4]. However, reaching a final diagnosis is still a challenging task, leading to an inconclusive diagnose for the majority of spontaneously abortion cases in ovine and bovine due to incorrect sampling or difficulties in the agent identification and serological testing [2,28]. Thus, investigating the etiologic cause of abortion is recommended for better understanding the causes of pregnancy issues and monitoring their occurrence. Therefore, expanding the search for pathogens and further identifying the causal agents of abortions is essential for implementing management and prevention measures to avoid economic losses in sheep and cattle industry due to pregnancy losses.

It was observed the occurrence of Cfv in 11.1% of the investigated bovine fetuses, while Cff was not detected in any sample. Worth mentioning that Cfv, despite being the causal agent of BGC, is rarely directly associated to abortions. A previous report [3] investigating the occurrence of *C. fetus* in natural cases of bovine and ovine abortions by immunohistochemical assay detected antigens for Cff in 50% ovine and in 4.5% bovine cases, while Cfv antigen was detected in bovine, totalizing 59% of bovine abortion cases. In South America, Cfv have been associated to 2.0% of aborted fetuses in Uruguay [15] and in 9.33% of bovine abortions in Argentina [18].

Moreover, *C. fetus* was not detected in none of the 12 ovine samples analyzed by PCR assay in the present study. Differently, a wide investigation of infectious agents associated with ovine abortion and stillbirths determined that *Campylobacter* spp. was associated to 10.3% of 1,784 cases, being Cff the most common species found (7.0%) [12]. Nevertheless, other etiologic agents of pregnancy losses can be involved in the abortion, such as *Toxoplasma gondii*, *Chlamydia psittaci*, *Trueperella pyogenes*, *Campylobacter jejuni*, *Salmonella* sp., *Pasteurella*, *Escherichia coli* [12]. Interestingly, although Cff is described as a commonly associated infectious agent that causes abortions in sheep, there is limited information about its occurrence in South America. In the last 2 decades, only few studies have investigated Cff occurrence and relationship with ovine abortion cases, being held in Brazil [9], Argentina [7], and Uruguay [6]. Furthermore, Cff is consider an opportunistic human pathogen, which may lead to diarrhea, bacteremia, and pregnancy issues as well [25]; therefore, further

investigation about the occurrence of this pathogen in cattle and ovine herd is required.

We highlight that some important causes of abortion in cattle could be excluded as causes, in this present study, because neither bacterial growth nor ruminant pestivirus identification were observed in both fetuses F1 and F2. Moreover, no microscopic finding indicative or pathognomonic of any infectious etiology was observed in fetuses F1 and F2. However, even those additional complementary results for agents such as *Leptospira* spp., *Toxoplasma* spp., *Chlamydia* spp., and *Neospora caninum* were not available; infection by these agents should be unlikely due to absence of lesions.

In both Cfv positive fetuses (F1 and F2), gross and microscopic evaluations were unremarkable. Pathological findings previously reported in cases of *Campylobacter fetus*-associated abortions include neutrophilic bronchopneumonia and interstitial pneumonia, fibrinosuppurative serositis, hepatitis, gastroenteritis, and neutrophilic and necrotic placentitis [3,17]. Nevertheless, macroscopic and microscopic findings are commonly not observed, being the pathological lesions associated with the high bacteria charge, which we could not presume in the studied cases. To the best of our knowledge, *C. fetus* is not a bacterium present in both fetuses and cow normal microbiota or without disease association; therefore, the molecular identification of *C. fetus* in aborted animals could be related to infectious abortion cases.

CONCLUSION

Our findings demonstrate de occurrence of *Campylobacter fetus* subsp. *venerealis* in spontaneously aborted cattle fetuses, illustrating its possible association in abortion cases in South Brazil. Overall, our results highlight the importance of investigating uncommon causal agents of abortions in sheep and cattle, in order to establish better prevention strategies to avoid infectious agents' circulation in the herd.

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