Concomitant infection of Neospora caninum and Bovine Herpesvirus type 5 in spontaneous bovine abortions

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ABSTRACT.- Marin M.S., Morrell E.L., Pérez S.E., Leunda M.R., Moore D.P., Jones L.R., Campero C.M. & Odeón A.C. 2013. Concomitant infection of Neospora caninum and Bovine Herpesvirus type 5 in spontaneous bovine abortions. Pesquisa Veterinária Brasileira 33(11):1291-1295. Área de Investigación en Producción y Sanidad Animal, Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Ruta 226 Km 73.5 (7620), Balcarce, Buenos Aires, Argentina. E-mail: aodeon@balcarce.inta.gov.ar

Bovine Herpesvirus type 5 (BoHV-5) has not been conclusively demonstrated to cause bovine abortion. Brain lesions produced by Neospora caninum and Bovine Herpesvirus type 1 (BoHV-1) exhibit common features. Therefore, careful microscopic evaluation and additional diagnostic procedures are required to achieve an accurate final etiological diagnosis. The aim of the present work was to investigate the occurrence of infections due to BoHV-1, BoHV-5 and N. caninum in 68 cases of spontaneous bovine abortions which showed microscopic lesions in the fetal central nervous system. This study allowed the identification of 4 (5.9%) fetuses with dual infection by BoHV-5 and N. caninum and 33 (48.5%) cases in which N. caninum was the sole pathogen identified. All cases were negative to BoHV-1. The results of this study provide evidence that dual infection by BoHV-5 and N. caninum occur during pregnancy in cattle; however, the role of BoHV-5 as a primary cause of bovine abortion needs further research. Molecular diagnosis of BoHV-5 and N. caninum confirmed the importance of applying complementary assays to improve the sensitivity of diagnosing bovine abortion.

INDEX TERMS: Abortion, Bovine Herpesvirus type 5, cattle, encephalitis, Neospora caninum.

INTRODUCTION

Bovine Herpesvirus type 1 (BoHV-1) and type 5 (BoHV-5) are two closely related alphaherpesviruses that infect cat-
These viruses belong to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Roizman & Pellett 2001). BoHV-1 is an important pathogen of cattle, causing significant economic losses to the cattle industry worldwide (Takiiuchi et al. 2005). It is responsible for a variety of clinical syndromes, including respiratory disease, conjunctivitis, abortion and genital infections. Sporadically, BoHV-1 is neuroinvasive and can cause encephalitis. On the other hand, BoHV-5 is a primary etiological agent of non-suppurative meningoencephalitis in calves (Pérez et al. 2002) and has occasionally been isolated from aborted bovine fetuses (Schudel et al. 1986). Both viruses have been implicated as causes of bovine abortion (Smith 1997). However, the role of BoHV-5 as an etiologic agent has not been conclusively demonstrated.

*Neospora caninum* is one of the most important agents causing abortion in cattle worldwide. Cows of any age may abort, from 3 months of gestation to term, with most abortions occurring at 5–6 months of gestation. Fetuses may die in the uterus, be reabsorbed, mummified, autolysed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected. This protozoan produces distinctive fetal lesions, particularly in the central nervous system (CNS), which consist of focal areas of necrotizing multifocal encephalitis and the presence of sporadic cysts and/or tachyzoites (Collantes-Fernández et al. 2006). On the other hand, considering that latent infection in the CNS may also occur even in calves without clinical signs, the finding of microscopic lesions are relevant for the diagnosis of *Neospora*-related abortion (Collantes-Fernández et al. 2006, Moore et al. 2008).

Brain lesions produced by *Neospora* and BoHV-1 exhibit common features (Brower et al. 2008). Therefore, careful microscopic evaluation and additional diagnostic procedures are necessary to achieve an accurate conclusive etiologic diagnosis. The aim of the present work was to investigate the occurrence of infections due to BoHV-1, BoHV-5 and *N. caninum* in 68 cases of spontaneous bovine abortions which showed microscopic lesions in the fetal CNS.

**MATERIALS AND METHODS**

Specimens from 383 aborted fetuses submitted for routine laboratory diagnosis of abortion from 2004 to 2010 were analyzed in this retrospective study. The fetuses were from beef and dairy herds of the “Humid Pampas” of Argentina. Diagnosis of bovine abortion was performed at the Animal Health Group, Veterinary Diagnosis Laboratories, National Institute of Agricultural Technology (INTA), Balcarce, Argentina. The routine laboratory methodology for the diagnosis of bovine abortion has been previously described by Campero et al. (2003).

For this study, 68/383 bovine fetuses were selected based on the histopathological lesions observed in the fetal CNS (non-suppurative meningitis, perivascular cuffings, hemorrhages, diffuse gliosis, focal gliosis, non-suppurative encephalitis and necrotizing encephalitis). For each fetus submitted for diagnosis, information on cattle production system (beef or dairy), age, sex and degree of autolysis was recorded. Tissues (lung, spleen, liver, kidney, lymph nodes) and abomasal content from all fetuses were collected for microbiological diagnosis by culture and/or a direct fluorescent antibody test (DFAT) for common reproductive pathogens (*Campylobacter fetus*, *Trichomonas foetus*, *Brucella abortus*, *Leptospira* spp.). Homogenates of spleen and lymph nodes were processed for viral isolation on Madin-Darby Bovine Kidney (MDBK) cells. After four blind passages of 48-72 h each, cultures were tested for bovine viral diarrhea virus (BVDV) and BoHV antigens by DFAT with a commercially available polyclonal antibody (American Bio-Research, Sevierville, TN, USA). Fetal tissues collected at necropsy were stored at -80°C for further analysis. For this study, DNA extracted from fetal CNS was analyzed by molecular biology procedures. Fetal fluids (thoracic and abdominal) collected at necropsy were tested by a serum neutralization test to determine the presence of specific antibodies to BoHV and BVDV (Odeón et al. 2001). Infection by *N. caninum* was evaluated by indirect fluorescent antibody test (IFAT) using dilution of fetal fluids at a ratio of 1:25 (Moore et al. 2002). Fetal tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE) for routine histological examination. CNS was also processed by immunohistochemistry (IHC) for detection of *Neospora caninum* (Campero et al. 2003) and BoHV-5 antigens (Brower et al. 2008). For this purpose, a BoHV-5-specific monoclonal antibody (LG6, VMRD, Pullman, WA, USA) (Chung et al. 1994) was used. IHC to BoHV-5 was only performed in fetuses which were BoHV-5 positive by polymerase chain reaction (PCR).

Table 1. Summary of the results obtained by serum neutralization test, IFAT, IHC and PCR for BoHV-5 and *Neospora caninum* in 68 spontaneously aborted bovine fetuses with microscopic lesions in the CNS

<table>
<thead>
<tr>
<th>Number of fetuses</th>
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*Serum neutralizing antibodies to BoHV, + − Negative, + Positive, nd = not determined.
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BoHV-1 (LA38 [accession number KC412237] and Cooper [accession number KC412238]) and with reference strains of other related viruses (Suid Herpesvirus type 1 [accession number EU719641.1] and Caprine Herpesvirus type 1 [accession number JX993260.1]). This analysis revealed that genomes detected in the four aborted fetuses are closely related to the BoHV-5 Argentinean reference strain (A663) (Fig.1). Three of the fetuses with dual infection belonged to beef herds; information on the procedence of the remaining fetus is lacking. Gestational ages of the BoHV-5 positive fetuses were 4, 6 and, 7 months (2 cases). Specific antibodies to *N. caninum* in fetal fluids were detected in 2 out of 4 fetuses with dual infection (Table 1). *N. caninum* was also identified by IHC in the CNS of 3 out of 4 aborted fetuses (Table 1). Due to the severe degree of autolysis, IHC could not be performed on one fetus. BoHV-5 was not detected by IHC in the CNS of dually infected fetuses. However, viral antigens were detected when IHC was performed on BoHV-5-infected MDBK cells (data not shown), demonstrating that other causes, likely related to tissue preservation, were involved in the lack of antigen detection.

Comparison of microscopic lesions from fetuses with dual infection and fetuses with solely *N. caninum*-infection were compared (Table 2). For this comparison, only the 4 fetuses which were consistently positive by IFAT, IHC and PCR were selected. Predominant microscopic lesions in fetal CNS are shown in Figure 2, 3 and 4. Focal necrotizing encephalitis was consistently observed in the fetuses in which only *N. caninum* infection was detected. Perivascular cuffings, gliosis, and multifocal non-suppurative encephalitis were also observed in the 3 fetuses with concomitant BoHV-5 and *N. caninum* infection. It was not possible to describe the CNS lesions in one fetus which had a high degree of autolysis.

**DISCUSSION**

Previous studies have demonstrated that, under optimal laboratory conditions, a final diagnosis of the cause of bovine abortion is possible. However, this study did not identify a single causal agent in all cases. It is possible that multiple pathogens were involved, or that the fetuses were infected simultaneously with multiple pathogens. Further research is needed to confirm these findings and to better understand the role of these agents in spontaneous bovine abortions.
abortion is only achieved in 25 to 45% of cases (Campero et al. 2006). In this work, the presence of microscopic lesions in the CNS of fetuses was suggestive of an infectious cause of abortion. Molecular diagnosis techniques, such as PCR, are important ancillary tests because they improve the sensitivity of the diagnosis, particularly when fetal autolysis is present.

Viral isolation from samples of abortions (fetuses and placenta) represents the “gold standard” for a conclusive diagnosis of BoHV infection. However, isolation is difficult, time-consuming and requires samples to be in optimal conditions to achieve the most favorable result. In fact, contamination, inadequate transportation conditions, autolysis, and other factors may all adversely affect viral isolation (Takiuchi et al. 2005). Routine diagnosis of Neospora caninum is based on histopathological changes in fetal tissues and identification of parasites by IHC. However, confirmation of N. caninum infection using IHC has low sensitivity (Baszler et al. 1999). In the present work, BoHV was not isolated, and N. caninum was identified by IHC only in 3 out of 4 aborted fetuses that presented a concomitant infection with BoHV-5, as detected by PCR. Moreover, in order to prove that BoHV-5 infection was the cause of abortion, viral antigen detection by IHC was also attempted. However, as expected, BoHV-5 was not detected in CNS sections. Tissue conditions were not optimal since fetal specimens had been long-term stored in formaldehyde, which is detrimental for antigen detection by IHC (Beckstead 1994). It is also likely that the use of a monoclonal antibody to detect BoHV-5 is highly specific but not sensitive enough, had also influenced the final outcome (Haines & Chelack 1991). Furthermore, fetal autolysis might also be responsible for the lack of antigen detection by this technique (Kirkbride 1986). Therefore, in these cases, the use of PCR is a more rapid and sensitive approach for diagnosis than virus isolation or IHC. Indeed, PCR should be considered a useful complementary tool for the diagnosis of both pathogens in fetuses with histopathological lesions in the CNS.

The prevalence of N. caninum found in this study (48.5%) is in agreement to that previously reported by Moore et al. (2008) by using PCR. Even though these data indicate the relevance of this parasite as a cause of bovine abortion, the presence of other pathogens cannot be excluded (Campero et al. 1998). Moreover, the presence of N. caninum DNA does not imply the parasite as the primary cause of abortion and microscopic observation of fetal tissues should be performed for the final diagnosis of Neospora-related abortion (Moore et al. 2008). On the other hand, pathological synergism by dual infections, like in these cases, has been previously hypothesized (Campero et al. 2003).

In the present work, BoHV-5 DNA was detected in fetuses with microscopic lesions in the CNS which were indicative of necrotizing meningoencephalitis and which were also in agreement with histopathological lesions described for fetal N. caninum-infections. The presence of BoHV-5 was confirmed by sequencing of PCR products and the corresponding phylogenetic analysis. The nucleotide sequence of the PCR-amplified gC gene fragment of 4 BoHV-5 cases was compared with the sequences of BoHV-1 and BoHV-5 reference strains. This analysis showed that viral genomes identified in the four aborted fetuses are closely related to...
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**REFERENCES**


