UNIVERSIDAD DE LA REPÚBLICA

FACULTAD DE VETERINARIA

Programa de Posgrados

CAUSAS DE ABORTO EN BOVINOS LECHEROS DE URUGUAY

MELISSA MACÍAS RIOSECO, MVZ, MPVM

TESIS DE DOCTORADO EN SALUD ANIMAL

URUGUAY

2018

ESTA HOJA VA EN BLANCO

UNIVERSIDAD DE LA REPÚBLICA

FACULTAD DE VETERINARIA

Programa de Posgrados

CAUSAS DE ABORTO EN BOVINOS LECHEROS DE URUGUAY

Melissa Macías Rioseco

Franklin Riet-Correa	**Federico Giannitti**
Director de Tesis	**Co-director**

2018

INTEGRACIÓN DEL TRIBUNAL DE

DEFENSA DE TESIS

Presidente: Dr. Rodolfo Rivero
Segundo miembro: Dr. Daniel Cavestany
Tercer Miembro: Dr. José M. Verdes

2018

ACTA DE DEFENSA DE TESIS

UNIVERSIDAD DE LA REPUBLICA
URUGUAY

FVET Facultad de Veterinaria Universidad de la República Uruguay
Programa de Posgrados

ACTA DE EXAMEN

CURSO: Defensa de Tesis de Doctorado

LUGAR Y FECHA DE LA DEFENSA: Montevideo, 12 de febrero de 2019

TRIBUNAL: Dr. Rodolfo Rivero (Presidente), Dr. Daniel Cavestany, Dr. José M. Verdes

CI ESTUDIANTE	NOMBRE	CALIFICACIÓN	NOTA
6.195.594-9	**MACÍAS RIOSECO, Melissa**	B.B.B.	06

PRESENTADOS	NO PRESENTADOS	APROBADOS	APLAZADOS	INSCRIPTOS
1	0	1	0	1

TRIBUNAL	FIRMA
Dr. Rodolfo Rivero (Presidente)	
Dr. Daniel Cavestany	
Dr. José M. Verdes	

ES COPIA FIEL

LEONARDO LANDIN
JEFE DE SECCIÓN
OFICINA DE POSGRADO

NOTA: Las calificaciones de aprobación de la Defensa de Tesis pueden ser:
B.B.B.- 6 o S.S.S.-12

En esta hoja va el Informe del Tribunal

UNIVERSIDAD DE LA REPUBLICA URUGUAY

FVET Facultad de Veterinaria Universidad de la República Uruguay
Programa de Posgrados

FACULTAD DE VETERINARIA
Programa de Posgrados

ACTA DE APROBACIÓN DE TESIS

DE DOCTORADO EN SALUD ANIMAL

"CAUSAS DE ABORTO EN BOVINOS LECHEROS DE URUGUAY"

Por: Melissa MACÍAS RIOSECO

Director de Tesis: Dr. Franklin Riet-Correa
Codirector de Tesis: Dr. Federico Giannitti

Tribunal

Presidente: Dr. Rodolfo Rivero

Segundo Miembro: Dr. Daniel Cavestany

Tercer Miembro: Dr. José M. Verdes

Fallo del Tribunal:

Montevideo, 12 de febrero de 2019

El Fallo de aprobación de la Tesis puede ser: Aprobada (corresponde a la nota BBB- en el Acta), o Aprobada con Mención (corresponde a la nota SSS- 12 en el Acta)

Agradecimientos:

A mi tutor Franklin Riet-Correa por darme la oportunidad de aprender a su lado y por guiarme en mi formación como investigadora y patóloga. Gracias por mostrarme como ser parte del cambio utilizando la ciencia como herramienta.

A mi cotutor Federico Giannitti, por enriquecer, con paciencia y con confianza, mi entrenamiento en diagnóstico, y así ayudarme a crecer profesionalmente.

Al DILAVE Montevideo, Institut Pasteur de Montevideo, al Centro Universitario Regional (CENUR) Litoral e INTA Balcarce por colaborar en mi formación.

Al INIA, ANII y a la Facultad de Veterinaria por darme la posibilidad de desarrollar mis estudios de posgrado.

A toda la gran familia de la Plataforma de Salud Animal, investigadores, estudiantes y técnicos, por su disposición, por las lecciones compartidas, por el excelente carácter y el compañerismo. A Caroline, por compartir esta aspiración.

A todos los estudiantes de grado y pasantes por su participación durante las necropsias de fetos.

A los veterinarios y a los productores por abrirme camino y por su confianza.

A mis siempre presentes: Gema, Issa, Diego, Valerie, Gabriela Caregnani, a la familia Caregnani Carro y mis hermanas chicalenses.

A mi familia: mi madre, mi padre, Elliot, Katya y Balta, sin ustedes jamás me hubiera lanzado.

A todos y todas, muchas, muchas gracias.

Índice

SUMMARY **11**

1. INTRODUCCIÓN Y ANTECEDENTES ESPECÍFICOS **12**

Referencias 14

2. PLANTEAMIENTO DEL PROBLEMA **15**

Referencias 16

3. HIPÓTESIS **16**

4. OBJETIVO **17**

5. INVESTIGACIÓN **17**

6.1. CAPÍTULO 1 ..... **19**

Causes of abortion in dairy cows from Uruguay 19

6.2. CAPÍTULO 2 ..... **39**

Bovine abortion due to *Coxiella burnetii*; report of a cluster of cases and review of the literature 39

6.3. CAPÍTULO 3 ..... **56**

Fetal pathology in an aborted Holstein fetus infected with bovine parainfluenza virus-3 genotype A (BPIV-3a, Respirovirus, Paramyxoviridae) 56

6.4. CAPÍTULO 4 ..... **67**

Abortion outbreak caused by *Campylobacter fetus* subspecies *venerealis* and *Neospora caninum* in a bovine dairy herd 67

7. CONSIDERACIONES FINALES **78**

RESUMEN

El diagnóstico del aborto bovino es complejo y, en muchos casos, no se logra la identificación de la causa. Reportes en Uruguay demuestran la circulación de varios agentes causales en ganado bovino lechero, e incluso la circulación de más de un agente abortivo en un mismo brote de abortos. Esto puede representar una dificultad adicional en el abordaje diagnóstico, particularmente en brotes. Se deben realizar estudios y una serie de pruebas para la observación de lesiones y la detección de distintos agentes patógenos en fetos abortados y placentas. Esta tesis doctoral es una serie de casos con el objetivo de determinar la frecuencia de las enfermedades que causan el aborto bovino en ganado lechero en Uruguay. El tamaño total de la muestra fue de 102 casos, compuestos por 53 fetos, 35 fetos y placentas y 14 placentas. La etiología del aborto se determinó en 38 (37%) casos. En los 38 casos con etiología determinada, 20 (50%) fueron causados por *Neospora caninum*, seis casos (16%) por *Coxiella burnetii* y dos (5%) por *Campylobacter fetus* subsp. *venerealis*. Dos casos se asociaron con infección por *Escherichia coli*. El virus Parainfluenza-3 bovino, *Salmonella enterica* serovar Newport y *Trueperella pyogenes* causaron un aborto cada uno. Otro aborto se asoció con una placentitis causada por una infección mixta por *Mannheimia* sp. y *Streptococcus* sp. En dos casos, la muerte de los fetos se debió a una distocia y en un caso el aborto fue causado por un mesotelioma congénito. Si bien el gen LipL32 se detectó en un homogenado de hígado en la PCR, este caso se clasificó como negativo para *Leptospira* spp. debido a la ausencia de lesiones en este feto. La PCR para el virus de la diarrea del virus bovino (BVDv) fue positiva en tres casos; dos de ellos estaban en coinfección con *Neospora caninum*, y se determinó que el protozoo era el agente causal. No se observaron lesiones en el otro feto infectado por BVDv. Dentro de los 64 casos sin diagnóstico etiológico, 27 casos (42%) de ellos tenían lesiones inflamatorias que sugerían un agente infeccioso. A pesar de la baja tasa de éxito en el diagnóstico de las causas del aborto en el ganado, el diagnóstico sistemático del aborto bovino es necesario para establecer posibles estrategias de prevención y control. Además, permite el monitoreo de enfermedades reproductivas en el ganado lechero, algunas de las cuales representan un riesgo para la salud pública.

Palabras cable: Aborto bovino, *Coxiella burnetii*, *Campylobacter* spp., Ganado lechero,

SUMMARY

The diagnosis of bovine abortion is complex and, in many cases, the identification of the cause is not achieved. Reports in Uruguay show the circulation of several causative agents of abortion in dairy cattle, and even the circulation of more than one abortive agent in the same abortion outbreak. This may represent an additional difficulty in the diagnostic approach, particularly in abortion outbreaks. There must be studies and a series of tests for the observation of lesions and the detection of different pathogens in aborted fetuses and placentas. This doctoral thesis is a case series study with the objective of determining the frequency of the diseases that cause bovine abortion in dairy cattle in Uruguay. The total sample size was 102 cases, consisting of 53 fetuses, 35 fetuses and placentas and 14 placentas. The etiology of abortion was determined in 38 (37%) cases. In the 38 cases with determined etiology, 20 (52.6%) were caused by *Neospora caninum*, six cases (15.7%) by *Coxiella burnetii* and two (5.2%) by *Campylobacter fetus* subsp. *venerealis*. Two cases were associated with infection by *Escherichia coli*. The bovine Parainfluenza-3 virus, *Salmonella enterica* serovar Newport and *Trueperella pyogenes* each caused an abortion. Another abortion was associated with placentitis caused by a mixed infection by *Mannheimia* sp. and *Streptococcus* sp. In two cases, the death of the fetuses was due to a dystocia and in one case the abortion was caused by a congenital mesothelioma. Although the LipL32 gene was detected in a liver homogenate in the PCR, this case was classified as negative for *Leptospira* spp. due to the absence of injuries in this fetus. The PCR for the bovine virus diarrhea virus (BVDv) was positive in three cases; two of them were in coinfection with *N. caninum*, and it was determined that the protozoan was the causative agent. No lesions were observed in the other fetus infected with BVDv. Among the 64 cases without an etiologic diagnosis, 27 cases (42%) of them had inflammatory lesions that suggested an infectious agent. Despite the low success rate in diagnosing the causes of abortion in cattle, the systematic diagnosis of bovine abortion is necessary to establish possible prevention and control strategies. In addition, it allows the monitoring and surveillance of reproductive diseases in dairy cattle, some of which represent a risk to public health.

Keywords: *Bovine abortion*, *Coxiella burnetii*, *Campylobacter* spp., Dairy Cattle,

1. INTRODUCCIÓN Y ANTECEDENTES ESPECÍFICOS

Las pérdidas de gestación que ocurren antes del día 45 post-concepción se consideran pérdidas embrionarias, mientras que son considerados abortos las pérdidas gestacionales seguidas de expulsión de un feto muerto, que ocurren entre el día 45 y el final de la gestación (Campero et al. 2018). En Uruguay, la producción de leche de origen bovino es una de las actividades pecuarias más importantes, después de la producción de carne. La industria ganadera en el mundo es afectada por varios problemas reproductivos como baja tasa de concepción, natimortos o abortos que causan pérdidas económicas importantes (De Vries 2006). El aborto en bovinos de leche también incrementa los costos de inseminación, tratamientos, alimentación y aumenta la tasa de descarte prematuro en animales de reemplazo (De Vries 2006).

Existe poca información sobre la prevalencia e incidencia de abortos en bovinos del cono sur. Corbellini y colaboradores examinaron en el 2006, 161 fetos abortados en el sur de Brasil y de lograron identificar la causa del aborto en el 51.5%. *Neospora. caninum* fue el agente más comúnmente responsable (23% de los casos), seguido por infecciones bacterianas en un 17.4%, hongos en un 3.1% y virus en un 1.8%. El estudio más reciente sobre causas de abortos en Argentina fue realizado en el 2010 por Morrell, en el periodo de 2005 al 2006, donde examinó 150 fetos de bovinos de producción ganadera y lechera, siendo en 69.2% y 23.4% respectivamente. El diagnóstico se obtuvo en el 52% de los casos, y de estos, las causas infecciosas fueron las más comunes (96%). Los patógenos *C. fetus*, *Leptospira* spp., *N. caninum* y *Brucella abortus* fueron los más frecuentes. En el resto de los casos que no se logró determinar el diagnóstico, pero en el 26 % de estos, se observaron lesiones histológicas sugestivas a una causa infecciosa.

En Chile se realizó un trabajo retrospectivo de 20 años, donde se analizaron 270 establecimientos ganaderos y un total de 494 fetos bovinos. El 48.8% de los casos fetos fueron de explotaciones lecheras, en comparación con 10.1 % de carne (el resto se indicó como de origen mixto). El diagnóstico etiológico se logró en el 59.7% de los casos y las causas infecciosas causaron aborto en un 52.2%. Los agentes infecciosos más comunes fueron *Leptospira* spp. en un 25.2%. En 22.1% se detectaron lesiones compatibles con *Neospora caninum*. *B. abortus* fue identificada como causa de aborto en 14.3%, herpes

virus bovino en 13.4% y diarrea viral bovina en 7.7% (Meyer Zarzar, 2013).

En Uruguay se analizaron 431 fetos bovinos abortados entre 2002 y 2004. La mayor proporción de los casos, el 54%, provenían de rodeos lecheros. En 241 fetos se detectó un agente infeccioso, siendo bacteriana la causa más común, seguido por virus, y parásitos. Los agentes detectados en mayor frecuencia fueron *Leptospira* spp. en 41% y *N. caninum* en el 36%. En un caso se reportó el aislamiento de simultaneo de *Tritrichomonas foetus* y *C. fetus*. La bacteria aislada con menor frecuencia fue *B. abortus* con un 3% de los casos. Por último, en el 23% de los casos analizados no se encontraron lesiones macroscópicas ni microscópicas (Easton, 2006).

El aborto bovino es un síndrome (Anderson et al., 1990; Campero et al., 2003, Gadicke y Monti, 2008), debido a la complejidad de sus etiologías. La gran variación de frecuencias de causas, en reportes previos de distintos lugares, muestran que puede ser debido a un difícil diagnóstico. Las muestras que deben ser remitidas para el diagnóstico de aborto incluyen el feto, la placenta, y sueros de las vacas que abortaron y de vacas que estuvieron en riesgo de abortar durante el mismo periodo de tiempo, pero que no abortaron. Pueden ser necesarias, también, muestras del medio ambiente, por ejemplo, alimentos, agua y/o plantas sospechosas de causar aborto.

El diagnóstico etiológico del aborto bovino es difícil por diversas razones. Por ejemplo, en la autolisis, que además de dificultar la observación de lesiones sutiles, la degradación del tejido puede causar la inactivación del agente e interferir en el aislamiento bacteriológico o viral. Algunas veces los cultivos se contaminan por bacterias oportunistas como *Escherichia coli*, *Mannheimia haemolytica*, *Truperella pyogenes*, *Bacillus* sp, *Pasteurella multocida*, entre otras (Clothier y Anderson, 2016).

Realizar un diagnóstico etiológico puede ser un desafío si no se cuenta con la metodología para descartar todos los agentes, incluyendo agentes poco comunes. Un estudio realizado en California, donde se evaluaron 665 fetos bovinos, indicó que aproximadamente el 10% de los fetos no presentaron lesiones específicas y no fue identificado ningún agente etiológico (Clothier y Anderson, 2016). En el mismo grupo de casos, en un 11.7% en los que se observaron lesiones macroscópicas y microscópicas (abomasitis, pleuritis, peritonitis, hepatitis, esplenitis miocarditis, encefalitis y timitis) no fueron identificados agentes infecciosos. Cuando se identifica una lesión, pero no se logra identificar el agente

infeccioso, la etiología puede ser un agente infeccioso poco reportado, ya que generalmente no se incluyen técnicas de diagnóstico correspondientes para su identificación. Entre los agentes poco diagnosticados se incluyen *Chlamydia abortus*, *Mycoplasma* spp., *Ureaplasma* spp. y *Coxiella burnetii*. Es importante tener un diagnóstico etiológico de los abortos en bovinos lecheros, para poder realizar una correcta estimación de pérdidas económicas de las diferentes causas de abortos y aumentar la producción de leche en Uruguay.

Referencias.

1. Anderson ML, Blanchard PC, Barr BC, & Hoffman RL.1990. A survey of causes of bovine abortion occurring in the San Joaquin Valley, California. J Vet Diagn Invest. 4, 283-287.
2. Campero CM, Cantón G, & Moore PD. 2018 In: Abortos y Otras Pérdidas Reproductivas en Bovinos. Diagnóstico y Control. Editorial Hemisferio Sur. 2018:31.
3. Clothier K., & Anderson M. 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology. 15:933-938.
4. Corellini LG, Pescador CA, Frantz F, Wunder E, SteffenD, Smith DR, & Driemier D. 2006. Diagnostic survey of bovine abortion with special reference to *Neospora caninum* infection: importance, repeated abortion and concurrent inecion in aborted fetuses in Southern Brazil. Vet J 172:114-120.
5. De Vries A. 2006. Economic value of pregnancy in dairy cattle. J Dairy Sci. 89(10):3876-3875.
6. Easton C. Estudio patológico de las principales causas infecciosas en el aborto bovino en Uruguay. Master´s thesis, Universidad de la República, Uruguay 2006.
7. Gadicke P & Monti G. 2008. Aspectos epidemiológicos y análisis del síndrome del aborto bovino. Arch eEd Vet 40: 223-224.
8. Meyer Zarzar. Estudio descriptivo de las principales causas de aborto bovino diagnosticados entre los años 1990 y 2010 en el Instituto de Patología Animal de la Universidad Austral de Chile. Tesis de grado. Universidad Austral de Chile, Valdivia Chile 2013.

9. Morrell E. Caracterización diagnóstica de las causas infecciosas del aborto bovino PhD Thesis Universidad Nacional de La Plata, Argentina 2010.

2. PLANTEAMIENTO DEL PROBLEMA

La producción de leche en Uruguay creció en los últimos años, alcanzando 2177 millones de litros en 2013 (DIEA 2013). Sin embargo, el número de bovinos lecheros se mantuvo relativamente estable (DICOSE 2014), lo que podría deberse, parcialmente, a una baja eficiencia reproductiva (Rovere 2007). El intervalo interpartos en 2008-2012 fue de 15 meses (INML 2014), y el intervalo parto-concepción aumentó de 150 días en 2001-2005 (Rovere 2007) a 172 días en 2008-2012 (INML 2014). Un incremento en la tasa de abortos podría ser responsable de este aumento. Existe escasa información sobre la incidencia de abortos en bovinos lecheros en Uruguay, aunque se estima que aproximadamente 8% de las hembras gestantes aborta (Dr. Carlos Grela -CONAPROLE-, comunicación personal). En una población de aproximadamente 320.000 vacas en ordeñe (DIEA 2013), esto representa una pérdida estimada de 25.600 fetos/año. Considerando que la frecuencia esperada de abortos en rodeos con buen manejo nutricional, sanitario y reproductivo es menor de 3-5% (Jonker 2004), una reducción de esta tasa de 8% a 5% representaría el nacimiento de 9.600 terneros más/año en el país. Las pérdidas económicas asociadas a los abortos incluyen pérdidas de terneros, pérdidas en producción de leche, los gastos reproductivos (protocolos de inseminación artificial, mano de obra, etc.), de alimentación y los posibles efectos negativos en la fertilidad futura de las hembras (Gädicke 2008)

El diagnóstico del aborto bovino es complejo y, en muchos casos, no se logra la identificación de la causa (Clothier y Anderson, 2016). La autólisis, además de dificultar la observación de lesiones sutiles, ocasiona la degradación del tejido que puede causar la inactivación del agente e interferir en el aislamiento bacteriológico o viral. Reportes en Uruguay sugieren la circulación de varios agentes causales de aborto bovino en ganado de leche, e incluso la circulación y acción de más de un agente abortivo en un mismo brote (Easton, 2006). Esto puede representar una dificultad adicional en el abordaje diagnóstico, particularmente en brotes de aborto. Se deben realizar estudios histológicos y una serie

de pruebas bacteriológicas, virológicas, inmunológicas y de biología molecular para la detección de distintos agentes patógenos en fetos y placentas.

Es necesario el diagnóstico sistemático de las causas de aborto, con la metodología correcta, para conocer las enfermedades abortivas, la epidemiología de estas y, posteriormente, determinar medidas de control adecuadas para disminuir las pérdidas económicas.

Referencias.

1. Clothier K., & Anderson M. 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology. 15:933-938.
2. DICOSE (2014). Datos de la Declaración Jurada de DOCISE 2012-datos de lechería.. [En línea]. Montevideo: MGAP. Consultado 26 de enero 2015. Disponible en: http://www.mgap.gub.uy/dgsg/DICOSE/Informe2013/DJ2013_TotalNacional.pdf.
3. DIEA (2013). Anuario Estadístico Agropecuario 2013. [En línea]. Montevideo: MGAP.246p. Consultado 11 noviembre 2014. Disponible en: http://www.mgap.gub.uy/ Dieaanterior/Anuario2011/DIEA-Anuario-2011-web.pdf.
4. Easton C. Estudio patológico de las principales causas infecciosas en el aborto bovino en Uruguay. Master´s thesis, Universidad de la República, Uruguay 2006.
5. Gädicke P., Monti, G. 2008. Aspectos epidemiológicos y de análisis del síndrome de aborto bovino. Arch. Med. Vet. 40: 223-234.
6. Jonker FH 2004. Fetal death: comparative aspects in large domestic animals. Animal Reproduction Science 82–83, 415–430.
7. Rovere G., Sotelo F., Valena J., Slavica J. 2007. Mejoramiento lechero y el monitoreo reproductivo de los tambos uruguayos [Cd-Rom]. En: IX Congreso Holstein de las Américas; Colonia, Uruguay.

3. HIPÓTESIS

El conocimiento de las causas y la frecuencia de abortos en ganado lechero permite establecer bases para crear estrategias de control, vigilancia y monitoreo de enfermedades abortivas en la cuenca lechera de Uruguay.

4. OBJETIVO

Determinar la frecuencia relativa de causas de aborto bovino en tambos del área de influencia del laboratorio de diagnóstico de la Plataforma de Salud Animal del Instituto Nacional de Investigación Agropecuaria.

5. INVESTIGACIÓN

La presente tesis consta de cuatro capítulos que fueron enviados a distintas revisas científicas con el fin de divulgar resultados asociados a este trabajo. En el primer capítulo se estudiaron una serie de casos donde se describe la frecuencia de distintos agentes abortigénicos presentes en fetos y placentas remitidos al laboratorio de diagnóstico de la Plataforma de Salud Animal del Instituto Nacional de Investigación Agropecuaria. El objetivo de este primer capítulo es describir las distintas frecuencias de los agentes presentes en la serie de casos. Este capítulo fue enviado en forma de articulo científico a la revista Pesquisa Veterinaria. El Capítulo 2 consta de un reporte de un brote de aborto por *Coxiella burnetii* y una revisión de literatura de coxielosis en bovinos. *Coxiella burnetii* se reporta usualmente como un agente causante de aborto esporádico en bovinos, sin embargo, durante la realización de esta tesis, pudimos identificar cuatro abortos causados por el patógeno durante un periodo reducido, provenientes de un mismo predio. Esta descripción resalta la importancia de evaluar varias muestras, además del feto, para lograr el diagnóstico de aborto bovino. Dicho capítulo fue remitido y publicado por la revista Journal of Veterinary Diagnostic Investigation. El tercer capítulo es el reporte de las lesiones fetales en un feto abortado por el virus de Parainfluenza Bovina genotipo A. Este reporte es el primero que se realiza y describe la particularidad de las lesiones fetales causadas por dicho virus, además de evidenciar la circulación del patógeno en poblaciones bovinas de Uruguay. Este capítulo fue remitido a la revista Veterinary Pathology y actualmente se encuentra en publicación. Por último, el Capítulo 4 es la descripción de un brote de abortos causadas por al menos dos patógenos distintos. En esta sección se discute la dinámica que varios agentes pueden tener en una determinada población. Describe un episodio epizoótico, donde se producen múltiples abortos durante un periodo de tiempo

específico en un predio. Se realizaron varias necropsias en distintos fetos, y se logró detectar *Campylobacter fetus* subsp. *venerealis* en un feto con lesiones características de este agente. En otros dos fetos se logran identificar lesiones compatibles a *N. caninum* y se detectó al agente en ambos casos. Este tipo de patrones epizoóticos marcan la importancia de realizar necropsias de varios fetos de un mismo predio, a pesar de contar con una historia previa de identificación de posibles agentes o de tener una previa sospecha de la causa, pues múltiples agentes pueden circular en el mismo predio y provocar abortos simultáneamente. Este último capítulo fue enviado, está aceptado y actualmente se encuentra en publicación, en la Revista Mexicana de Ciencias Pecuarias.

CAPÍTULO 1: Causes of abortion in dairy cows in Uruguay

Melissa Macías-Rioseco[2,3], Caroline Silveira[2,3], Martin Fraga[2], Laura Casaux[2,3], Andrés Cabrera[4], María E. Francia[5], Carlos Robello[4], Leticia Maya[6], Leticia Zarantonelli[7,8], Alejandra Suanes[9], Rodney Colina[6], Alejandro Buschiazzo[8], Federico Giannitti[2] and Franklin Riet-Correa[2*]

Instituto Nacional de Investigación Agropecuaria (INIA). Plataforma de Salud Animal. Estación Experimental INIA La Estanzuela. Ruta 50 km 11, 39173, Colonia, Uruguay. E-mail: frcorrea@inia.org.uy

Received on ……..……

Accepted for publication on ……

[2]Instituto Nacional de Investigación Agropecuaria (INIA). Plataforma de Salud Animal. Estación Experimental INIA La Estanzuela. Ruta 50 km 11, 39173, Colonia, Uruguay. *Corresponding Author: frcorrea@inia.org.uy

[3]Programa de Posgrado en Salud Animal. Universidad de la Republica, Facultad de Veterinaria. Montevideo, Uruguay

[4]Unidad de Biología Molecular. Institut Pasteur de Montevideo, Montevideo, Uruguay

[5]Laboratory of Apicomplexan Biology. Institut Pasteur de Montevideo, Montevideo, Uruguay

[6]Laboratorio de Virología Molecular, Centro Universitario Regional Litoral Norte, Universidad de la República, Salto, Uruguay.

[7]Unidad Mixta Pasteur+ Instituto Nacional de Investigación Agropecuaria, Institut Pasteur de Montevideo, Montevideo, Uruguay.

[8]Laboratorio de Microbiología Molecular y Estructural, Institut Pasteur de Montevideo, Montevideo, Uruguay.

[9]División de Laboratorios Veterinarios. Ministerio de Ganadería, Agricultura y Pesca, Montevideo, Uruguay.

Abstract

A case series study was conducted to determine the frequency of causes of abortion in dairy cattle in Uruguay. The sample size of 102 cases was composed of 53 fetuses, 35 fetuses with placentas, and 14 placentas without an associated fetus. All cases underwent gross and microscopic pathologic examinations as well as microbiological and serological serological testing. The etiology was determined in 54 (53%) of cases, 51 of which were caused by infectious agents. Within the observed 102 cases, 30 (29%) were caused by *Neospora caninum*, six (6%) by *Coxiella burnetii* and two (2%) by *Campylobacter fetus* subsp. *venerealis*. Bovine Parainfluenza-3 virus and *Salmonella enterica* serovar Newport each caused one abortion each. Opportunistic bacteria (*Escherichia coli*, *Streptococcus*sp., *Staphylococcus* sp, *Mannheimia* sp., *Trueperella pyogenes*, and *Providencia stuartii*) were associated with 11 abortions. In two cases the fetal death was attributed to dystocia, and in one case the fetus had a congenital mesothelioma. Bovine viral diarrhea virus (BVDV) infection was identified in three fetuses; two of which were co-infected with and had typical lesions of *N. caninum*. No lesions were observed in the other fetus infected by BVDV. *Leptospira interrogans* was identified in one fetus without lesions. Despite the relatively low overall success rate in establishing an etiological diagnosis in cases of abortion in cattle, a systemic workup of bovine abortion is necessary to establish prevention and control strategies. This also facilitates monitoring and surveillance of reproductive diseases in dairy cattle, some of which represent a risk to public health.

INDEX TERMS: Bovine abortion, *Campylobacter fetus*, *Coxiella burnetii*, dairy cattle, *Neospora caninum*.

Introduction

In Uruguay, dairy production is one of the most important economic activities of the agricultural sector. Abortion in dairy cattle increases the cost of reproduction, medical treatments, feeding, and culling and replacement rates (De Vries 2006). Abortions are gestational losses that occur between day 45 of pregnancy and the end of gestation (Campero et al. 2018). There is little information available about the prevalence of abortion in dairy cattle in South America. A study from Brazil examined 161 aborted bovine fetuses from Rio Grande do Sul and the cause of abortion was determined in 51.5% of the cases (Corbellini et al. 2006). *Neospora caninum* was the most commonly detected agent (23% of the cases) followed by bacteria in 17.4%, fungi in 3.1% and viruses in 1.8% (Corbellini et al. 2006). The most recent study from Argentina, examined 150 bovine fetuses between 2004 and 2006 (Morrell 2010). The studied population was composed of dairy (23.4%) and beef (69.2%) cattle; the production class was unknown in the remaining cases (7.4%). The diagnosis was determined in 52% of the cases with *N. caninum* (14.7%), *Campylobacter fetus* (9.3%), *Leptospira* spp. (7.3%), and *Brucella abortus* (6.7%) being the most common agents. An etiological diagnosis was not achieved in the remainder 48% of the cases, although 25.3% of these had microscopic lesions suggestive of an infectious cause (Campero et al. 2018).

In Chile, a longitudinal study of 20 years examined 270 farms and examined a total of 494 bovine fetuses. Forty-eight percent of cases were from dairy farms, 10.1% from beef farms, and the rest from mixed production farms. The etiology was determined in 59.7% of the cases, and 52.2% of the cases had an infectious cause. The most commonly identified agent was *Leptospira* spp. in 25.2%. About 22% of the cases had microscopic lesions compatible with *N. caninum*, and *B. abortus* was detected in 14.3%. The two main viral agents reported were bovine herpesvirus 1 in 13.4% and bovine viral diarrhea virus (BVDV) in 7.7% of the infectious cases (Meyer Zarzar 2013). Infectious etiologies are among the most commonly reported abortigenic agents in cattle in Uruguay. A study conducted from 2002 to 2004 analyzed 431 aborted bovine fetuses from dairy (54%) and beef (46%) farms. In 41% of the cases with diagnosis, the abortion was attributed to leptospirosis based on the detection of leptospiral maternal antibodies, and *N. caninum* was identified in 36%. *Campylobacter fetus* was reported in 13% of the cases, one of which, based on microscopic observation, was determined as coinfected with

Tritrichomonas foetus. The bacterial etiology with the lowest reported frequency was *B. abortus* in 3% of the cases. Lastly, about 23% of the examined fetuses did not have any macroscopic or microscopic lesions and were of undetermined cause (Easton 2006). The diagnosis of bovine abortion is complex and the diversity of causes cannot be identified despite extensive laboratory testing (Antoniassi et al. 2013, Clothier & Anderson 2016). Bovine abortion has been considered a syndrome because of the complexity of their causes (Anderson et al. 1990, Campero et al. 2003). Fetal and placental autolysis is common, precludes the observation of common, obscuring lesions and interfering with the successful identification of infectious agents. The circulation of more than one abortigenic agent in the same herd could represent another difficulty in the diagnosis of bovine abortion, particularly in epidemic outbreaks (Macías-Rioseco et al. 2019). Likewise, bacterial cultures from fetal tissues and placentas are frequently contaminated with non-pathogenic or opportunistic bacteria (Clothier & Anderson 2016), making interpretation of results difficult, particularly in cases without lesions typical of bacterial infection. Nonetheless, a plethora of diagnostic approaches such as necropsy, histology, immunohistochemistry, bacteriology, virology, immunology and molecular biology assays, aid in the diagnosis of bovine abortion.

Achieving the etiologic diagnosis in bovine abortion is challenging mainly when adequate diagnostic tests are not available, particularly when caused by infrequent or opportunistic agents, or agents that are nonculturable by traditional microbiological methods. A study of 655 cases of bovine abortion in California, revealed that about 20% of the examined fetuses did not have any specific lesions (Clothier & Anderson 2016). Moreover, about 11.7% of these cases had macroscopic and/or microscopic lesions (abomasitis, pleuritis, peritonitis, hepatitis, splenitis, myocarditis, encephalitis and thymitis) with no pathogens identified. When a lesion is identified but there is no etiology found, infectious agents that are rarely detected in cases of abortion or difficult to isolate should be considered. The ideal diagnostic tests may be unavailable or perhaps the appropriate samples are not submitted to the laboratory for testing. Ideal samples for bovine abortion investigation are the aborted fetus, placenta, and serum from aborted and matched pregnant non-aborted dams. The objective of this work was to identify and determine the relative frequency of etiologies in cases of abortion in dairy cattle submitted to a veterinary diagnostic laboratory in Uruguay from 2015 to 2018.

Materials and methods

Case selection and case definition

From January 1st, 2015 to November 1st, 2018, bovine abortion cases were processed at the veterinary diagnostic laboratory of the Platform of Animal Health, at the National Institute of Agricultural Research in La Estanzuela experimental station, Colonia, Uruguay. Cases were either submitted to the laboratory by veterinary practitioners or collected directly from the dairy farms by our team upon the practitioner's request. Cases were defined as: 1) an aborted fetus; 2) an aborted fetus with its placenta; and 3) a placenta from an animal that aborted. Cases submitted from the same farm at the same time and composed of two or more fetuses and/or placentas from different dams were categorized as different cases. The etiologic diagnosis was determined by the association of the identified pathogen and the presence of compatible gross and/or microscopic lesions (see section on diagnostic tests below). Cases with an identified pathogen but with no lesions, and cases with lesions but with no causative agent identified were categorized as abortion cases of undetermined etiology. Information on sex, date of submission, geographic location of the farm, breed and age were obtained from the veterinarians and farmers. The fetal age in days was estimated based on the crown-to-rump length and other gross characteristics of the fetuses, and further categorized in first, second or third trimester of gestation. Cases were also categorized by the degree of autolysis as mild, moderate or severe.

Pathology examination

Macroscopic evaluation of the placentas and fetuses were done by veterinary laboratory diagnosticians with pathology training. Samples of liver, kidneys, adrenal glands, spleen, lymph nodes, thymus, lungs, heart, brain, skeletal muscles, fore-stomachs, abomasum, cecum, spiral colon, duodenum, jejunum, ileum, and placenta (when available), were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-6 µm, and stained with hematoxylin and eosin for microscopic examination. Depending on the initial microscopic examination and assessment of lesions and/or results of other diagnostic tests, and immunohistochemical (IHC) procedures for the detection of *Coxiella burnetii*, *Chlamydia* spp., BVDV, bovine parainfluenza virus 3, and/or *N. caninum* were performed in selected tissues in some cases (see below). At necropsies, samples were collected for bacteriology, serology, molecular biology or immunology testing (see below).

Diagnostic tests

Placenta, liver, lung, and abomasal fluid from all cases were inoculated onto blood and MacConkey agars (Oxoid, Basingstoke, Hampshire, England) and incubated at 37°C in aerobic conditions. In addition, samples were also inoculated onto Skirrow agar (Oxoid, Basingstoke, Hampshire, England) and incubated in anaerobic jars (CampyGenTM, Oxoid, Basingstoke, Hampshire, England), at 37°C for 2 to 7 days in an atmosphere of 5-10% of oxygen and 5-10% carbon dioxide (Chaban et al. 2013). In the cases when bacterial isolates were obtained, bacterial identification was performed either by routine biochemical test or by sequencing the16S rRNA. Placenta, liver, kidney, aqueous humor, and abomasal fluid were also spiked into Leptospira Medium Base Ellinghausen, McCullough, Johnson and Harris (EMJH) and incubated in aerobiosis at 29°C for up to 6 months (Zarantonelli et al. 2018). Fetal pericardial fluid (when available) was analyzed by microagglutination test (MAT) to detect antibodies against *Leptospira* serovars Grippotyphosa, Icterohaemorrhagiae, Pomona, Canicola, Hardjo Bovis, Hardjo Prajitno and Wolfii at a cutoff point of 1/10 (Zarantonelli et al. 2018).

PCR for the lipL32 gene of pathogenic *Leptospira* species was done from the 102 cases, as homogenates of liver, kidney and, placenta (when available). The primers LipL32F (5'-ATCTCCGTTGCACTCTTTGC-3') and LipL32R (5'-ACCATCATCATCATCGTCCA-3') were used. The mix was exposed at 95°C for 5 min, then 35 cycles of 30 sec at 95°C, for denaturation, and 30 sec at 58°C, 60 sec at 72°C, and 7 min at 72°C for annealing and extension (Zarantonelli et al. 2018).

Direct immunofluorescence (DIF) for *Campylobacter fetus* was done on impression smears from all placentas, abomasal fluid, lung and liver. Smears were fixed in ethanol at room temperature and incubated with an anti-*Campylobacter* antibody conjugated with fluorescein isothiocyanate (FITC) (Biotandil, Tandil, Buenos Aires, Argentina) (Figueiredo et al. 2002). Similarly, DIF for *Leptospira* spp. was performed on impression smears from kidney, liver, aqueous humor and placenta using LEP-FAC multivalent rabbit FITC-bound antibody (NVSL, Ames, Iowa, USA). The smears were visualized in a fluorescence microscope (AxioLab.A1, Carl-Zeiss, Germany) set at wavelengths of 495 nm excitation and 519 nm emission. Additionally, abomasal fluid or placenta smears were examined directly under dark field microscopy to assess for agents

morphologically compatible with *Campylobacter* spp., *Leptospira* spp. and *Tritrichomonas foetus*. PCR for bovine herpesvirus-1 (BHV-1) and reverse transcriptase PCR for BVDV were performed in pools of liver, lung, spleen, kidney, thymus, heart, brain, placenta, lymph node, and adrenal gland obtained individually in all cases that included fetuses. For BVDV, the primers V324mod (5'-ATGCCCWTAGTAGGACTAGCA-3') and V326mod (5' -WCAACTCCATGTGCCATGTAC-3') were used based on Maya (2016). Briefly, the mix was exposed at 95°C for 10 min, then 45 cycles of 10 sec at 95°C, for denaturation, and 60 sec at 50°C for annealing and extension. The primers gCBoHV F (5'-GCGGGGGCTCGCCGAGGA-3') and gCBoHV R (5'-GGAGCGCACGGTCAGGGGC-3') were used for BHV-1 PCR. The mix was exposed at 95°C for 5min, then 35 cycles of 30 sec at 95°C, for denaturation, and 30 sec at 60°C for annealing, 1 min at 72°C for extension and lastly, 10 min at 72°C (Silva et al. 2007). The IHC for BVDV antigen detection was done only in cases with a positive RT-PCR result for this virus. Antigen retrieval was done with heat in a decloaking chamber (Biocare Medical). A mouse IgG anti-BVDV (VMRD, Pullman, WA) was applied as primary antibody, followed by anti-mouse IgG horseradish peroxidase (HRP)-labeled polymer (DAKO, Santa Clara, CA), and 3-amino-9-ethylcarbazole (AEC, DAKO, Santa Clara, CA) as the chromogen. In one fetus with pneumonia with syncytial cells, RT-PCR for bovine parainfluenza virus 3 (BPIV-3) was done from frozen samples of lung and the amplification products were sequenced, as we have previously described for this same case (Macías-Rioseco et al. 2019). Based on the presence of compatible lesions (necrotizing and suppurative placentitis with intratrophoblastic bacteria), IHC for *C. burnetii* and *Chlamydia* spp. were done in selected cases (Dilbeck & McElwain 1994, Giannitti et al. 2016). When a positive result for *C. burnetii* by IHC was obtained, PCR was used for detection of this pathogen in the formalin-fixed paraffin-embedded (FFPE) blocks containing placenta (Lorenz et al. 1998). The procedures for *Chlamydia* spp. IHC and *C. burnetii* IHC and PCR in FFPE placenta in these same cases has recently been published by our group (Macías-Rioseco et al. 2019). Immunohistochemistry and PCR for detection of *N. caninum* antigen and DNA, respectively, were done only in cases with compatible histologic lesions in the brain, heart, placenta and/or liver. For the IHC, a goat polyclonal antibody (VMRD, Pullman, WA) against *N. caninum* was used as a

primary antibody, anti-goat IgG horseradish peroxidase (HRP)-labeled polymer (Vector polymer enzyme detection kit, Burlingame, CA) as the secondary antibody, and 3-amino-9-ethylcarbazole as the chromogen (DAKO, Santa Clara, CA). The PCR for *N. caninum* was done following the procedure described by Yamage (1996). PCR products were visualized on 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, USA), purified with QIAquick PCR Purification Kit and sequenced in house at the sequencing service of the Institut Pasteur de Montevideo, Uruguay. All tests were performed with appropriate positive and negative controls for each run.

Statistical Methods

The results of the pathologic examinations and diagnostic tests, as well as the fetal gestational age, degree of autolysis and the department where the dairy farm was located were recorded in a database using Microsoft Excel, and descriptive statistics were obtained.

Results

The series was composed of 102 cases; 53 of the cases were only fetuses, 35 were placentas with fetuses, and 14 were placentas only. The 102 cases were submitted from 45 different farms. The maximum number of cases submitted from the same farm was 28. Most of the cases were submitted from the department of Colonia with 58 cases, followed by San Jose (17), Canelones (6), Lavalleja (4), Soriano (2), Florida (2) and Rio Negro (2). The department was not recorded in 11 cases. The degree of autolysis was recorded in 95 cases: 59 had mild autolysis, 28 had moderate autolysis, and eight were severely autolyzed, five of these were mummified fetuses for which no etiology was determined. Forty-two fetuses were in the second trimester of gestation at the time of the abortion, followed by 33 cases in the third trimester and five in the first trimester.

The etiology of the abortion was determined in 54 (53%) cases, while the cause was undetermined in 48 (47%). Of the cases with undetermined etiology, 11 (23%) had inflammatory and/or necrotizing lesions in various tissues suggesting an infectious process, although no agent could be identified in the tissues by the set of diagnostic tests performed in the study. Of the 54 cases that had an etiologic diagnosis, 51 (94.4%) were caused by infectious agents. Thirty-eight of the 51 (74.5%) were caused by agents that are primarily recognized as reproductive pathogens, one was caused by PI3 virus and another by *Salmonella enterica* serovar Newport. The remainder 11 cases (21.5%) were

associated with opportunistic bacteria. The 38 cases caused by reproductive pathogens included *N. caninum* (30 cases), *C. burnetii* (6 cases), and *C. fetus* subsp. *venerealis* (2 cases). Of the 11 cases caused by opportunistic pathogens, *Escherichia coli* was identified in 4 cases, *Streptococcus* spp. in 2 cases, and *Streptococcus pyogenes*, *Staphylococcus* sp.,*Trueperella pyogenes*, *Providencia stuartii*, and *Mannheimia* sp. were identified in one case each. Placentitis, bronchopneumonia, and/or hepatitis were the main histologic lesions observed in association with these opportunistic agents. Regarding the three cases with non-infectious causes (5.6% of the 54 cases with an identified cause), in two cases the death of the fetuses was due to dystocia and in one case the abortion was caused by a congenital mesothelioma affecting the abdominal and pelvic viscera that was extensive enough to be considered incompatible with life. The percentage of diagnosis was variable according to the material sent to the laboratory. When only the fetus was sent, the diagnosis was made in 50.9% of the cases; when we received the fetus and placenta the diagnosis was made in 62.8% of the cases, and when only the placenta was available the diagnosis was made in 37.7% of the cases (Table 1).

Table 1. Percentages of cases with diagnosis and without diagnosis within type of sample submitted

Sample submitted	With diagnosis	Without diagnosis	Total
Fetus	27 (50.9%)	26 (49.1%)	53
Fetus and placenta	22 (62.8%)	13 (37.2%)	35
Placenta	5 (35.7%)	9 (66.3%)	14
Total	54	48	102

The 30 abortions caused by *N. caninum* (55.6% of the 54 cases with determined etiology) were from 17 different dairy farms, accounting for 37.8% of the 45 farms included in the study. Of the 6 abortions caused by *C.burnetii*, five were from the same farm. *Campylobacter fetus* subsp. *venerealis* was the etiology in 2 cases from different farms.

The number of cases submitted per farm varied from one to 28, and the number of causes of abortion diagnosed on each farm varied from one to four (Fig. 1).

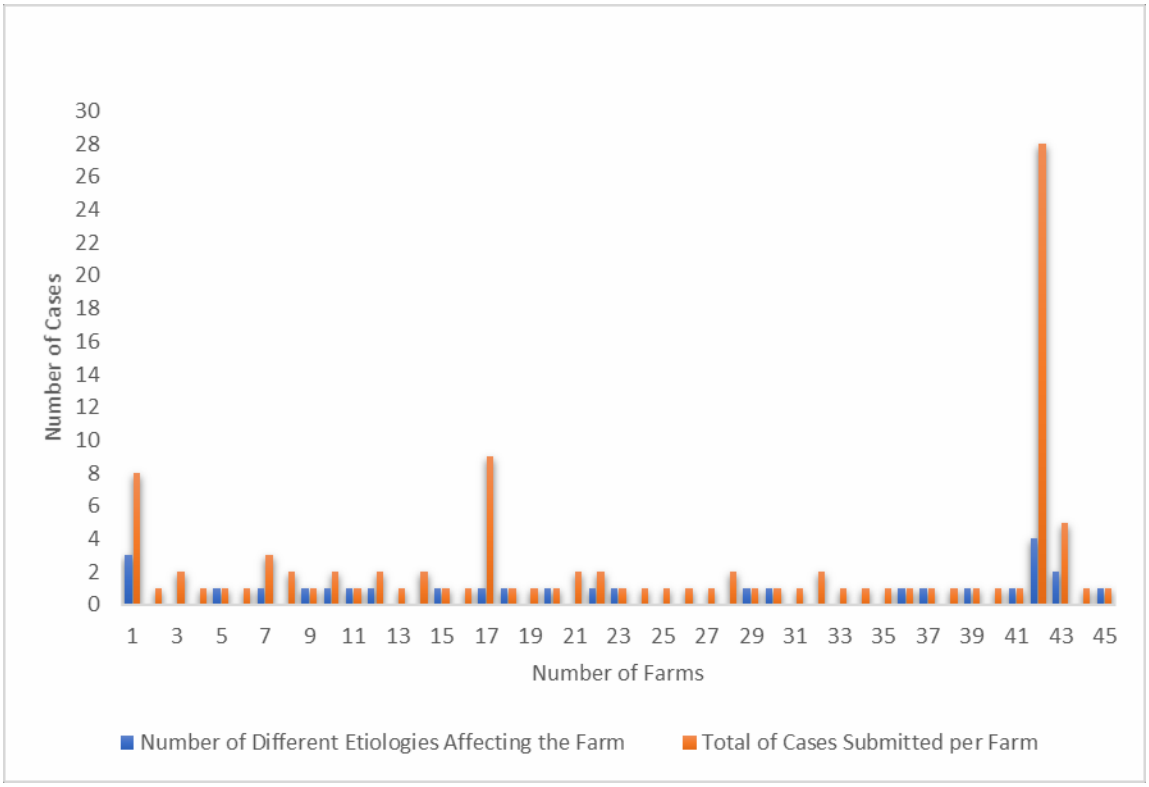


Figure 1. Frequency of different etiologies affecting each farm and total number of cases submitted per farm.

In farm 42, four different etiologic agents causing abortion were identified: *N. caninum* in two cases, *C. burnetii* in five, *Salmonella enterica* serovar Newport in one case, and BPiV-3 in another. In farm 43, five aborted fetuses were sent to the laboratory during an abortion outbreak, one abortion was caused by *C. fetus* subsp. *venerealis* and two by *N. caninum*; in the other two fetuses the cause of abortion was undetermined (Macías-Rioseco et al. 2019). In farm 1, one abortion was caused by *N. caninum*, another by *E. coli*, and one was due to dystocia. In farm 17, an etiologic diagnosis could only be confirmed in one of nine examined cases. The cases caused by *N. caninum* corresponded to abortions within the second trimester of gestation. The 30 fetuses aborted by neosporosis had typical lesions including non-suppurative encephalitis and gliosis (19 fetuses), myocarditis (15), hepatitis (9), interstitial pneumonia (5) and/or interstitial nephritis (3). Of the 30 fetuses with diagnosis of neosporosis, 18 showed encephalitis.

The six abortions caused by *C. burnetii* corresponded to full-term gestations and had moderate to severe multifocal necrotizing and suppurative placentitis with intralesional

and intratrophoblastic bacteria, and only one case had mild neutrophilic alveolitis. No cases of coxiellosis were diagnosed in cases where no placenta was available for examination. One case of abortion due to *Campylobacter fetus* subsp. *venerealis* had a non-suppurative fibrinous epicarditis and myocarditis; the placenta was not available for examination. The other case of *C. fetus* subsp. *venerealis* abortion had suppurative placentitis with arteriolitis and fibrinoid necrosis, neutrophilic bronchiolitis and alveolitis along with neutrophilic and histiocytic portal hepatitis. Suppurative and necrotizing placentitis was observed in cases associated with *E. coli*, *T. pyogenes*, and *S. enterica* serovar Newport. The latter also had intralesional coccobacilli in the placenta, along with minimal to mild neutrophilic lymphadenitis. In one case caused by *Staphylococcus* sp., the agent was isolated from the skin and from abomasal fluid, the fetus had diffuse hyperkeratosis, neutrophilic, histiocytic and fibrinous synovitis, along with moderate non-suppurative interstitial pneumonia with neutrophilic alveolitis and mild non-suppurative meningoencephalitis. One case caused by *Mannheimia* sp. had a moderate lymphohistiocytic and neutrophilic placentitis with multifocal trophoblastic necrosis. BPIV-3 caused multifocal neutrophilic and histiocytic alveolitis (pneumonia) with a moderate number of syncytial cells in the lungs and intestines in one case (Macías-Rioseco et al. 2019).18 In cases with lesions but without identified etiology, the lesions consisted of myocarditis, myositis, glossitis, gliosis, nephritis, pneumonia, and hepatitis.

No *B. abortus* was isolated in any case. Other bacterial agents such as *Acinetobacter lwofii*, *Aerococcus urinae*, *Providencia* sp., *Yersinia* sp., *E. coli*, *Enterobacter* sp., *Corynebacterium* sp. and *Serratia* sp. were isolated on bacterial cultures, but due the absence of associated lesions expected for bacterial infections, these abortions were classified as of undetermined etiology. The gene lipL32 of *Leptospira* spp. was detected in one sample of liver by PCR, the causality of the abortion was not attributed to this agent based on the lack of fetal lesions generally associated with leptospirosis. None of the tested fetal pericardial and/or thoracic fluids were reactive at the cutoff reference point for MAT for *Leptospira* spp. antibodies, including the case that was PCR-positive for *Leptospira interrogans*. *Leptospira* spp. culture was negative in all cases. PCR for BHV-1 was negative in all cases. RT-PCR for BVDV was positive in three cases; two of them were in co-infection with *N. caninum*, and the protozoon was determined as the causal

agent based on the presence of typical lesions and the positive results for *N. caninum* PCR and IHC. Due the absence of lesions, the etiology on the third case was categorized as undetermined. Two of the three BVDV PCR-positive fetuses were aborted in the second trimester and the other in the third trimester of gestation. To assess whether these fetuses congenitally infected with BVDV harbored high antigenic viral loads and thus were persistently infected, IHC for BVDV antigen detection was performed in several tissues (brain, lung, heart, thymus, liver and small intestine), with negative results in all three cases (data not shown). Based on these results, we interpreted that the fetuses were suffering from transient BVDV infections.

Discussion

In this case series, the most common cause of abortion was *N. caninum*. A previous study in Uruguay, showed a seroprevalence of *N. caninum* of 22% in dairy cows and 92% of the herds (Piaggio 2006). Our series was composed mainly of fetuses of gestational age in the second trimester, which may be explained in part by the high frequency of neosporosis. Abortions due to *N. caninum* are most commonly seen during the second and third trimesters of gestation (McAllister 2016). Our results and the high proportion of farms with seropositive cattle to *N. caninum* suggest that abortions caused by this agent potentially occur in most farms (Silveira 2019). *Coxiella burnetii* is rarely reported as a cause of abortion in cattle (Agerholm 2014). In our study, this agent was observed as a sporadic cause of abortion (only one case in one dairy farm) and as a cluster of five cases within a period of five months in another farm (Macías-Rioseco et al. 2019). We were able to identify abortions due to coxiellosis based on the microscopic evaluation of the placenta followed by IHC along with PCR for *C. burnetii*. In all cases, the diagnosis of coxielosis was based on the presence of necrotizing and suppurative placentitis with intralesional and intratrophoblastic bacteria identified as *C. burnetii*. The presence of lesions associated with the bacteria is key to the diagnosis of coxiliosis (Bildfell et al. 2000, Hazlett et al. 2013) since *C. burnetti* can be identified by PCR in the placenta of ruminants without being the cause of abortion (Hazlett et al. 2013).

These results suggest that *C. burnetii* is a previously undiagnosed, cause of abortion in dairy cattle in Uruguay, and highlight the importance of examining the placenta in aborted cattle to achieve this diagnosis. The presence of antibodies against *C. burnetii*

in slaughterhouse workers from Uruguay has been associated with history of clinical signs, and a clinical case of endocarditis (Moreira et al. 1987), showing that this agent is an occupational hazard for veterinarians and slaughterhouse workers. Also, antibodies against *C. burnetii* have been found in different animal species in this country, including cattle (Moreira et al. 1987).

Campylobacter fetus subsp. *venerealis* was observed in relative low frequency (2 cases of 54 with an identified etiology), which could be due the fact that this venereally transmitted agent is usually a cause of infertility and causes abortion only sporadically (Michi et al. 2016, Silva et al. 2007). Abortions are more commonly detected between the fourth and sixth months of gestation (Silveira et al. 2018). A national survey that evaluated 340 dairy farms identified that about 50% of the farms use only natural breeding with bulls, and an additional 20% use a combination of natural breeding and artificial insemination (INALE 2014). This suggest that campylobacteriosis may still be a problem in dairy farms in Uruguay.

As seen in this study, several causes of abortion were diagnosed in some farms, which indicates that the diagnosis of a primary cause of abortion, including neosporosis, campylobacteriosis, or coxiellosis, does not exclude other causes of abortion. Hence, it is recommended to attempt the diagnosis in as many cases as possible in order to increase the chances of detecting other abortifacients and better understand the situation of each farm. In our case series, only one of the tested fetuses was positive for *Leptospira* spp. by PCR on a liver sample Sequencing confirmed that the infection corresponded to the species *L. interrogans*. Fetal pericardial and/or thoracic fluids were antibody-negative by MAT in all fetuses, including in the PCR-positive one. DIF and/or dark field microscopy for the detection of leptospires were also negative in all fetal imprints and abomasal fluids. While *Leptospira* spp. cannot be excluded as a relevant abortigenic pathogen in cattle in Uruguay, our study did not allow for confirmation of *Leptospira* spp. as a cause of abortions following the diagnostic criteria and case definition we established. Many *Leptospira* species and serovars have recently been isolated from bovine urine and blood samples in Uruguay, including *L. interrogans* serogroup Pomona serovar Kennewicki (20 strains), *L. interrogans* serogroup Canicola serovar Canicola (1 strain), *L. borgpetersenii* serogroup Sejroe serovar Hardjo (10 strains) and *L. noguchii* (9 strains, belonging to a variety of serogroups) (Zarantonelli et al. 2018). According to that study,

20% of the almost 1,000 sampled cows were eliminating *Leptospira* spp. in the urine (Zarantonelli et al. 2018). It is striking that with such a high number of animals eliminating leptospiras in the urine, the present study did not find abortions caused by leptospirosis. It cannot be ruled out that *Leptospira*-induced abortions might occur with no detectable infection of the fetus or placenta and be caused by other mechanisms including the pathogen-triggered inflammatory cascade (Raghupathy 2001, Zi et al. 2015). Examples of such abortigenic scenarios include subclinical infections in the mother due to urinary tract infection (Schieve et al. 1994), periodontitis (Zi et al. 2015), bacterial vaginosis (Giakoumelou et al. 2016), among other pathologies. In animals such inflammatory processes linked to premature birth or miscarriage have also been described e.g. in bovine viral diarrhea (Moennig & Liess 1995), suggesting that different pathogen strains can produce different clinical outcomes. The role of the different *Leptospira* species found in the urine of healthy cattle as a cause of sporadic abortions and other reproductive failures should thus be investigated (Zarantonelli et al. 2018). In the present series of abortions, most cases corresponded to sporadic abortions and only few outbreaks were studied. It is possible that infections by some *Leptospira* species and/or specific serovars may cause abortion outbreaks in herds, which probably were not subjected to confirmatory laboratory investigation during the period of this study. Establishing a surveillance system to study abortion outbreaks in Uruguay seems important to better understand the pathogenesis, epidemiology and best diagnostic techniques for leptospirosis and other abortive diseases in the country. Viral abortions in cattle are reported in low frequencies in several studies (de Almeida Vaucher et al. 2011, Clothier & Anderson 2016). In this case series, one abortion was caused by BPIV-3. This agent had been previously isolated from a bovine fetus and the case diagnosed in our study has been published elsewhere (Macías-Rioseco et al. 2019). Abortions due to BHV-1 and BVDV were not observed in this series, although BVDV RNA was detected in three fetuses. None of the three cases had lesions compatible to BVDV, and IHC reactivities for BVDV antigen in liver, small intestine and brain were negative. The molecular detection of the pathogen confirms the circulation of the virus in these herds. Even though it seems that BVDV was not responsible for the abortions in these cases, it is important to test affected herds to identify persistently infected animals and the possibility of the occurrence of other forms of BVDV-associated diseases in the farms. Two of the positive BVDV animals were also positive for *N. caninum* by PCR

and/or IHC and in fact had tissue lesions consistent with neosporosis, that was considered the primary cause of the abortions. It has been suggested that BVDV infections allow other pathogens to easily cross the fetoplacental barrier, increasing the risk of abortion (Murray 1991, Quinn et al. 2004).

One abortion in our series was due to *Salmonella* Newport, which is rarely reported causing abortions (Campero et al. 2018). In this farm the serovar Newport also caused neonatal calf diarrhea and neonatal mortality due to septicemia during the same period (data not shown), indicating that the abortion was part of the spectrum of diseases typically associated with salmonellosis in dairy cattle and not an isolated event. In cattle, *Salmonella enterica* serovars Typhimurium, Dublin and Newport are the most commonly cause of salmonellosis. The clinical disease can be enteritic and/or septicemic, the latter can result in abortion in pregnant cattle (Uzal et al. 2016, Campero et al. 2018, Costa et al. 2018). Abortions due to *Salmonella* are mostly associated with *Salmonella* Dublin (Campero et al. 2003), less frequently, with *S*. Typhimurium (Easton 2006).

Other bacteria such us *E. coli*, *Streptococcus* spp., *T. pyogenes*, *Staphylococcus* spp. and *Mannheimia* spp. have previously been recognized as sporadic abortifacients in cattle, and as in other reports, were associated with suppurative lesions in the placenta, lungs, and occasionally other fetal tissues (Anderson et al. 1990, Campero et al. 2003, Clothier & Anderson 2016, Campero et al. 2018). One case was attributed to a congenital neoplasia (mesothelioma). While congenital neoplasia in cattle is very uncommon, mesothelioma is within the most frequently diagnosed cancers in bovine fetuses. Although mesotheliomas are of mesenchymal origin, they should be differentiated from disseminated metastatic adenocarcinomas. In adults the occurrence of mesotheliomas has been associated with exposure to asbestos, while this is less clear in fetuses (Peli et al. 2018).

Conclusions

Neosporosis is the main cause of abortions in the dairy cattle population that we studied. Coxiellosis is a cause of outbreaks or sporadic cases of abortions in Uruguay. Campylobacteriosis is still a cause of abortions. It is important to further investigate the pathogenesis mechanisms, epidemiology and diagnosis of leptospirosis to determine the importance of this disease as a cause of abortion in Uruguay. The systematic diagnosis of bovine abortion is necessary to set possible strategies of prevention and control, besides monitoring and surveillance of reproductive diseases in dairy cattle, some of which can

represent a risk to public health.

Acknowledgments.- This research was financially supported by Agencia Nacional de Investigación e Innovación (ANII), grant FSSA_X_2014_1_105696. The authors thank Yisell Perdomo, Cecilia Monesiglio, Anderson Saravia, Bruno Lopez, and all graduate students from the animal health platform at INIA, Jennifer McKenna from UW, Francisco Uzal, Karen Sverlow and Juliann Beingesser from CAHFS-UC Davis for valuable assistance. We are grateful to Luis Corbellini, for his keen and valuable suggestions preparing the manuscript, Ximena Salaberry from the "Division de Laboratorios Veterinarios" (DILAVE) of the Uruguayan "Ministerio de Ganadería, Agricultura y Pesca" (MGAP), Santiago Mirazo from UdelaR, and Cecilia Nieves and Camila Hamond from the Institut Pasteur, Montevideo.

References.

1. Agerholm J.S. 2014. *Coxiella burnetii* and reproductive disorders in cattle. Infect. Genet. Evol. 28:150.

2. Anderson M.L., Blanchard P.C., Barr B.C. & Hoffman R.L. 1990. A survey of causes of bovine abortion occurring in the San Joaquin Valley, California. J. Vet. Diagn. Invest. 4:283-287.

3. Antoniassi N.A.B., Juffo G.D., Santos A.S., Pescador C.A., Corbellini L.G. & Driemeier D. 2013. Causas de aborto bovino diagnosticadas no Setor de Patologia Veterinária da UFRGS de 2003 a 2011. Pesq. Vet. Bras. 69:69-72.

4. Bildfell R. J., Thomson G. W., Haines D. M., Beverly J. M. & Smartet N. 2000. *Coxiella burnetii* infection is associated with placentitis in cases of bovine abortion. J Vet Diagn. Invest. 12:419–425

5. Campero C.M., Moore D.P., Odeón A.C., Cipolla A.L. & Odriozola E. 2003. Aetiology of bovine abortion in Argentina. Vet. Res. Commun. 27(5):359-69.

6. Campero C.M., Cantón G. & Moore D.P. 2018. Abortos y otras pérdidas reproductivas en bovinos. Diagnóstico y control. 1st ed. Editorial Hemisferio Sur, Buenos Aires, Argentina, p. 41-46.

7. Chaban B., Garcia Guerra A., Hendrick S.H., Waldner C.L. & Hill J.E. 2013. Isolation rates of *Campylobacter fetus* subsp. *venerealis* from bovine preputial samples via passive filtration on nonselective medium versus selective medium, with and without transport medium. Am. J. Vet. Res. 74:1066-1069.

8. Clothier K. & Anderson M. 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 15:933-938.

9. Corbellini L.G., Pescador C.A., Frantz F., Wunder E., Steffen D., Smith D.R. & Driemeier D. 2006. Diagnostic survey of bovine abortion with special reference to *Neospora caninum* infection: importance, repeated abortion and concurrent infection in aborted fetuses in Southern Brazil. Vet. J. 172:114-120.

10. Costa R.A., Casaux M.L., Caffarena R.D., Macías-Rioseco M., Schild C.O., Fraga M., Riet-Correa F. & Giannitti F. 2018. Urocystitis and ureteritis in Holstein calves with septicaemia caused by *Salmonella enterica* serotype Dublin. J. Comp. Pathol. 164:32-36.

11. de Almeida Vaucher R., Dezen D., Simonetti A.B., Spilki F.R., & Roehe P.M. 2011. Phylogenetic characterization of bovine parainfluenza 3 from contaminated cell cultures and field isolates from Brazil. Braz. J. Microbiol. 42:1440–1444.

12. De Vries A. 2006. Economic value of pregnancy in dairy cattle. J. Dairy Sci. 89(10):3876-3875 Dilbeck P.M. & McElwain T.F. 1994. Immunohistochemical detection of *Coxiella burnetii* in formalin-fixed placenta. J.
13. Vet. Diagn. Invest. 6:125-127.

14. Easton C. 2006. Estudio patológico de las principales causas de aborto bovino en Uruguay. Masters Thesis, Universidad de la República, Montevideo.

15. Figueiredo J.F., Pellegrin A.O., Fóscolo C.B., Machado R.P., Miranda K.L. & Lage A.P. 2002. Evaluation of direct fluorescent antibody test for the diagnosis of bovine genital campylobacteriosis. Rev. Latinoam. Microbiol. 44:118-123.

16. Giakoumelou S., Wheelhouse N., Cuschieri K., Entrican G., Howie S.E. & Horne A.W. 2016. The role of infection in miscarriage. Hum. Reprod. Update. 22(1):116-133.

17. Giannitti F., Anderson M., Miller M., Rowe J., Sverlow K., Vasquez M. & Cantón G. 2016. *Chlamydia pecorum*: fetal and placental lesions in sporadic caprine abortion. J. Vet. Diagn. Invest. 28:184-189.

18. Hazlett M.J., McDowall R., DeLay J., Stalker M., McEwen, B., van Dreumel T., Spinato M., Binnington B., Slavic D., Carman S. & Cai H.I. 2013. A prospective study of sheep and goat abortion using real-time polymerase chain reaction and cut point estimation shows *Coxiella burnetii* and *Chlamydophila abortus* infection concurrently with other major pathogens. J Vet Diagn Invest 2013; 25:359–368

19. INALE. 2014. Instituto Nacional de la Leche. Encuesta Lechera. Inale 2014. Disponible en <http://www.inale.org/innovaportal/v/3597/2/innova.front/ encuesta-lechera-inale-2014.html> Accessed 21 December 2018.

20. Lorenz H., Jäger C., Willems H., & Baljer G. 1998. PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. Appl. Environ. Microbiol. 64:4234-4237.

21. Macías-Rioseco M., Caffarena R.D., Fraga M., Silveira C., Giannitti F., Cantón G., Hecker H.P., Suanes A. & Riet-Correa F. 2019. Abortion outbreak caused by *Campylobacter fetus* subspecies venerealis and *Neospora caninum* in a bovine dairy herd. Revista Mexicana de Ciencias Pecuarias 10 (4):1055-1063

22. Macías-Rioseco M., Mirazo S., Uzal F.A., Fraga M., Silveira C., Maya L., Riet-Correa F., Arbiza J., Colina R., Anderson M.L. & Giannitti F. 2019. Fetal pathology in an aborted Holstein fetus infected with bovine parainfluenza virus-3 genotype A. Vet. Pathol. 56(2):277-281.

23. Macías-Rioseco M., Riet-Correa F., Miller M.M., Sondgeroth K., Fraga M., Silveira C., Uzal F.A. & Giannitti F. 2019. Bovine abortion caused by *Coxiella burnetii*; report of a cluster of cases and review of the literature. J. Vet. Diagn. Invest. (4):634-639.

24. Maya L., Puentes R., Reolón E., Acuña P., Riet F., Rivero R., Cristina J.& Colina R. 2016. Molecular diversity of bovine viral diarrhea virus in Uruguay. Arch Virol. 161:529-535.

25. McAllister M.M. 2016. Diagnosis and control of bovine neosporosis. Vet. Clin. North Am. Food Anim. Pract. 32(2):443-463.

26. Meyer Zarzar L.P. 2013. Estudio descriptivo de las principales causas de aborto bovino diagnosticados entre los años 1990 y 2010 en el Instituto de Patología Animal de la Universidad Austral de Chile. Masters Thesis, Universidad Austral de Chile, Santiago, Chile.

27. Michi A.N., Favetto P.H., Kastelic J. & Cobo E.R. 2016. A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. Theriogenology. 85(5):781-791.

28. Moennig V. & Liess B. 1995. Pathogenesis of intrauterine infections with Bovine Viral Diarrhea Virus. Vet. Clin. North Am. Food Anim. Pract. 11(3):477-487.

29. Moreira R.E.S., Caffarena R.M., Perez G., Somma S. & Monteiro M. 1987. Fiebre "Q" en Uruguay. Rev. Inst. Med. Trop. S. Paulo. 29:168-173.

30. Morrell E. 2010. Caracterización diagnóstica de las causas infecciosas del aborto bovino. PhD Thesis, Universidad Nacional de La Plata. Argentina.

31. Murray R.D. 1991. Lesions in aborted bovine fetuses and placenta associated with bovine viral diarrhea virus infection. Arch. Virol. 3; (Suppl 1) S:217–224.

32. Piaggio J. 2006. Estudio transversal de neosporosis en la principal cuenca lechera del Uruguay. Masters Thesis, Universidad de la República, Montevideo Uruguay.

33. Peli A., Bolcato M., Roccaro M., Gentile A. & Militerno G. 2018. Mesothelioma in cattle: two case reports. Large Animal Review (24):89-92.

34. Quinn H.E., Windsor P.A., Kirkland P.D. &Ellis J.T. 2004. An outbreak of abortion in a dairy herd associated with *Neospora caninum* and bovine pestivirus infection. Aust. Vet. J. 82(1-2):99-101.

35. Raghupathy R. 2001. Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. Semin. Immunol. 13(4):219-227.

36. Schieve L.A., Handler A., Hershow R., Persky V. & Davis F. 1994. Urinary tract infection during pregnancy: its association with maternal morbidity and perinatal outcome. Am. J. Public Health 84(3):405-410.

37. Silva M.S., Brum M.C., Loreto E.L., Weiblen R. & Flores E.F. 2007. Molecular and antigenic characterization of Brazilian bovine herpesvirus type 1 isolates recovered from the brain of cattle with neurological disease. Virus Res. 129(2):191-199.

38. Silveira C.S., Fraga M., Giannitti F., Macías-Rioseco M., & Riet-Correa F. 2018. Diagnosis of bovine genital campylobacteriosis in South America. Front. Vet. Sci. 5:321.

39. Silveira C.S. 2019. Enfermedades infecciosas que causan abortos en bovinos con enfoque en rodeos lecheros de Uruguay. PhD Masters Thesis, Universidad de la República, Montevideo.

40. Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p. 167-176. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

41. Yamage M., Flechtner O. & Gottstein B. 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). J. Parasitol. 82(2):272-279.

42. Zarantonelli L., Suanes A., Meny P., Buroni F., Nieves C., Salaberry X., Briano C., Ashfield N., Da Silva Silveira C., Dutra F., Easton C., Fraga M., Giannitti F., Hamond C., Macías-Rioseco M., Menéndez C., Mortola A., Picardeau M., Quintero J., Ríos C., Rodríguez V., Romero A., Varela G., Rivero R., Schelotto F., Riet-Correa F. & Buschiazzo A. 2018. Isolation of pathogenic *Leptospira* strains from naturally infected cattle in Uruguay reveals high serovar diversity and uncovers a relevant risk for human leptospirosis. PLoS Negl. Trop. Dis. 12:9.

43. Zi M.Y., Longo P.L., Bueno-Silva B.& Mayer M.P. 2015. Mechanisms Involved in the Association between Periodontitis and Complications in Pregnancy. Front. Public Health. 2:290.

CAPÍTULO 2:

Bovine abortion due to *Coxiella burnetii*; report of a cluster of cases and review of the literature

Melissa Macías-Rioseco, Franklin Riet-Correa, Myrna M. Miller, Kerry Sondgeroth, Martin Fraga, Caroline Silveira, Francisco A. Uzal, Federico Giannitti

Plataforma de Investigación en Salud Animal, Instituto Nacional de Investigación Agropecuaria, La Estanzuela, Colonia, Uruguay (Macías-Rioseco, Riet-Correa, Fraga, Silveira, Giannitti). California Animal Health and Food Safety, Laboratory, University of California at Davis, San Bernardino, CA, USA (Uzal). Wyoming State Veterinary Diagnostic Laboratory, University of Wyoming, Laramie, WY, USA (Miller, Sondgeroth). Veterinary Population Medicine Department, University of Minnesota, Saint Paul, MN, USA (Giannitti).

Corresponding Author: F.A. Uzal. California Animal Health and Food Safety Laboratory, University of California at Davis, 105 W Central Ave, San Bernardino, CA 92408. E-mail: fuzal@ucdavis.edu

Abstract

A cluster of four bovine abortions by *Coxiella burnetii* in a dairy herd during a period of three months is described. Case 1 consisted of a placenta from an aborted cow; cases 2, 3 and 4 were fetuses and their placentas. Grossly, the placenta of one aborted cow had moderate, diffuse reddening of the cotyledons and loss of translucency of the intercotyledonary areas. No gross lesions were observed in the other three placentas. Microscopically the four placentas showed fibrinonecrotizing placentitis, and abundant intratrophoblastic gram negative coccobacilli were observed in two of the placentas. *C. burnetii* was identified intralesionally by immunohistochemistry (IHC) in all four placentas, and by PCR and DNA sequencing in three placentas analyzed by these techniques. One fetus had mild neutrophilic alveolitis with multinucleated syncytial cells; no gross or microscopic lesions were observed in the other two fetuses examined. The lungs of the three fetuses were negative for *C. burnetii* by IHC. All the tests performed to investigate other possible causes of abortions in the four cases were negative. *C. burnetii* causes Q fever in humans and animals. Until now, clusters of abortions in cattle by *C. burnetii* have not been reported; instead, this bacterium has been considered an opportunistic pathogen associated only with sporadic abortion in cattle. These findings indicate that *C. burnetii* may cause clusters of abortions in cattle and a complete analysis of the placenta is necessary for the diagnosis of *C. burnetii* abortion.

Keywords: Bovine abortion, *Coxiella burnetii*, coxiellosis, Q fever, zoonosis

Coxiella burnetii is a gram negative, intracellular proteobacterium of worldwide distribution that causes Query (Q) fever in humans and animals.[2,3,25] Infection by *C. burnetii* has been reported in a variety of mammals, including, but not limited to cattle, sheep, goats, buffaloes, pigs, horses, pacific harbor seals and rodents.[28,40] Several avian and invertebrate species can also be infected.[7] Q fever is a zoonosis; people that work with animals or who drink raw milk are mostly at risk.[32] In humans, Q fever can produce hepatitis, pneumonia, endocarditis and abortions, mainly in immunocompromised individuals,[44] but a large proportion of human infections are either subclinical or undiagnosed, because clinical signs are usually non-specific.[43] In ruminants, Q fever is mainly subclinical, but anorexia, stillbirth, and late term abortion have been described.[3] Here we present a cluster of four bovine abortions due to *C. burnetii* in a dairy farm during a period of two months and review the literature on Q fever in cattle. The four cases studied (cases 1, 2, 3, 4) originated from a pasture-based *Brucella abortus*-free dairy farm in the department of Colonia, Uruguay, between April and June 2017. Case 1 consisted of a placenta from an aborted American Holstein biotype cow; cases 2, 3 and 4 included fetuses and the placentas of two American Holstein biotype cows (cases 2 and 4) and one New Zealand biotype cow (case 3).

The affected dairy herd was composed of 356 Holstein cows, including 279 of the American biotype and 77 of the New Zealand biotype. The latter had been introduced to the herd between March and May 2017 from three different commercial dairy farms. The gestational age of the three fetuses examined was estimated to be 240-270 days based on crown-to-rump length, presence or absence of hair on lips, eyebrows and muzzle, eruption of incisor teeth, and presence or absence of horn pit. In case 1, the abortion occurred in the third trimester of gestation, although the fetus was not available to estimate the gestational age more precisely. Gross examination of the placentas and full autopsies of the fetuses were performed. All fetuses and placentas were in mild state of post-mortem decomposition. Grossly, the placenta from case 1 showed moderate, diffuse reddening of the cotyledons and loss of translucency of the intercotyledonary areas (Fig. 1). No gross lesions were observed in the other placentas and fetuses.

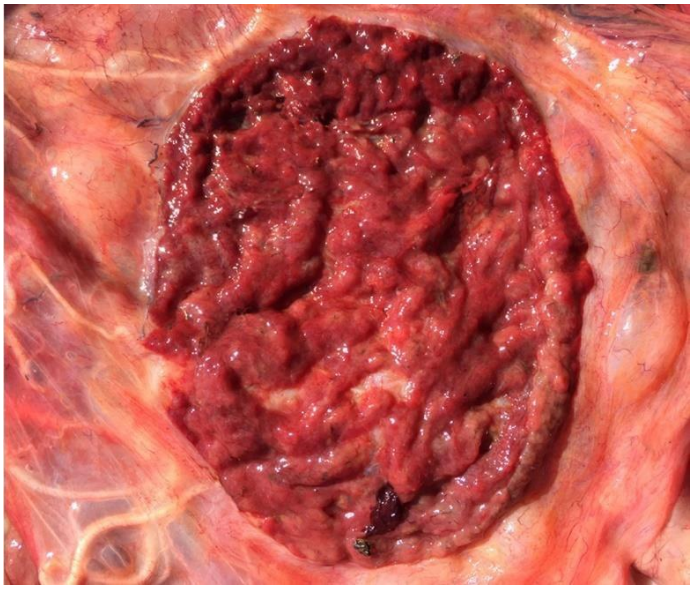


Figure 1. Bovine placenta, aborted cow, case 1. The cotyledon (center) shows moderate and diffuse reddening and the intercotyledonary area (periphery) depicts dullness and loss of normal translucency.

Samples of placentas and fetal tissues, including lung, liver, kidney, brain, spleen, thymus, lymph nodes, heart, tongue, skeletal muscle and gastrointestinal tract, were fixed in 10% neutral buffered formalin (pH 7) for 48 h, dehydrated through graded alcohols to xylene, embedded in paraffin, cut at 4-6 microns, and stained with hematoxylin and eosin. Selected sections were stained with gram.

Microscopically, the four placentas showed fibrinonecrotizing placentitis consisting of abundant neutrophilic infiltrate, necrotic debris and trophoblastic cells cytoplasmic basophilia compatible with intracytoplasmic bacteria with intralesional bacteria affecting cotyledons. In two of the placentas, large numbers of gram negative cocco-bacilli were seen in the cytoplasm of trophoblasts (Fig. 2 and 3).

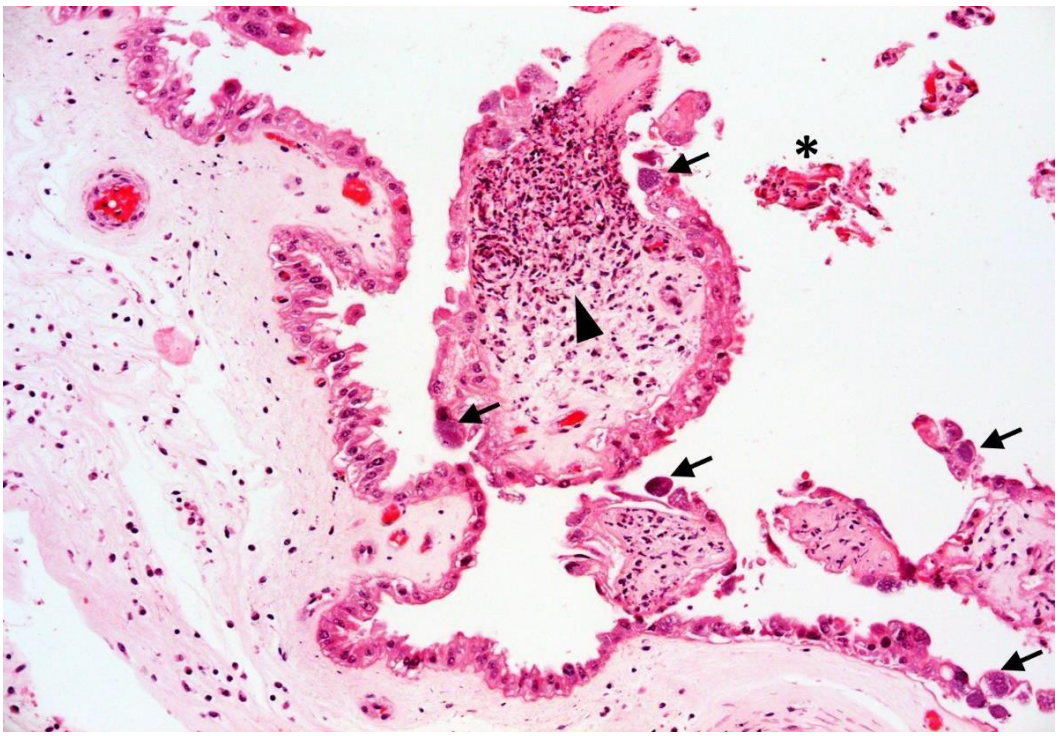


Figure 2. Cotyledonary placenta from aborted cow, case 4, stained with hematoxylin and eosin. The stroma underlying a chorionic villus is expanded by abundant neutrophilic infiltrate and necrotic debris (severe necrosuppurative placentitis, arrowhead). Trophoblastic cells adjacent and distant to the inflammatory reaction show increased cytoplasmic basophilia (arrows) due to intracytoplasmic bacteria, as depicted in Fig. 3. Sloughed necrotic trophoblastic cells are indicated with an asterisk.

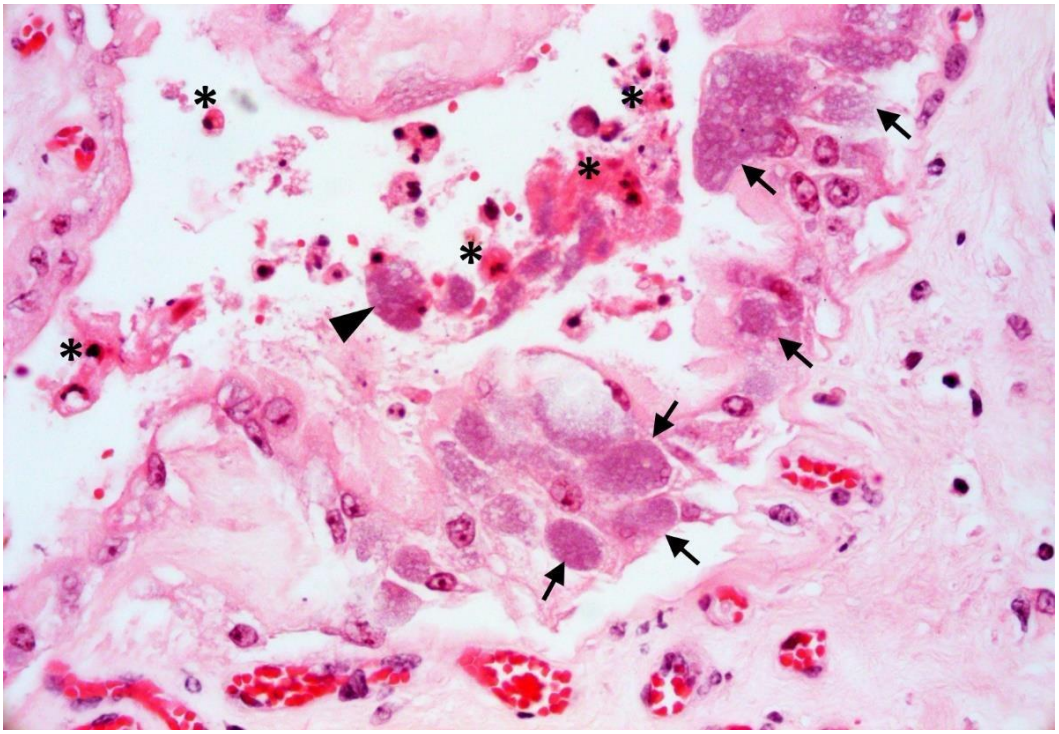


Figure 3. Cotyledonary placenta from aborted cow, case 4, stained with hematoxylin and eosin. Clusters of basophilic bacteria expand the cytoplasm of numerous trophoblasts (arrows), some of which have a pyknotic nucleus (necrosis) displaced to the periphery of the cell and are detached from the stroma (arrowhead). Sloughed hypereosinophilic and karyorrhectic trophoblastic debris are indicated with asterisks.

Diffuse edema was observed in the intercotyledonary area. The fetus in case 3 had mild neutrophilic alveolitis with multinucleated syncytial cells within the alveolar spaces. No other significant microscopic lesions were observed in the rest of the tissues of any of the the other fetuses or in the placentas.

Selected sections of the four placentas and lungs from the three fetuses were processed by immunohistochemistry (IHC) for *C. burnetii* and *Chlamydia* spp.,[22] according to standard operating procedures of the California Animal Health and Food Safety Laboratory, UC Davis. Human *Coxiella burnetii*-positive (JW261M1-2) was used, primary antibodies. y and the analysis was performed with appropriate negative and positive controls.[19]

IHC for *C. burnetii* revealed abundant intralesional antigen in the four placentas (Fig. 4), although the sections of lung of the three fetuses were negative for this test. The immunoreaction was localized diffusely in the chorionic villi; within the trophoblast,

as granular intracytoplasmic immunomarcation. *Chlamydia* spp. IHC was negative in all samples.

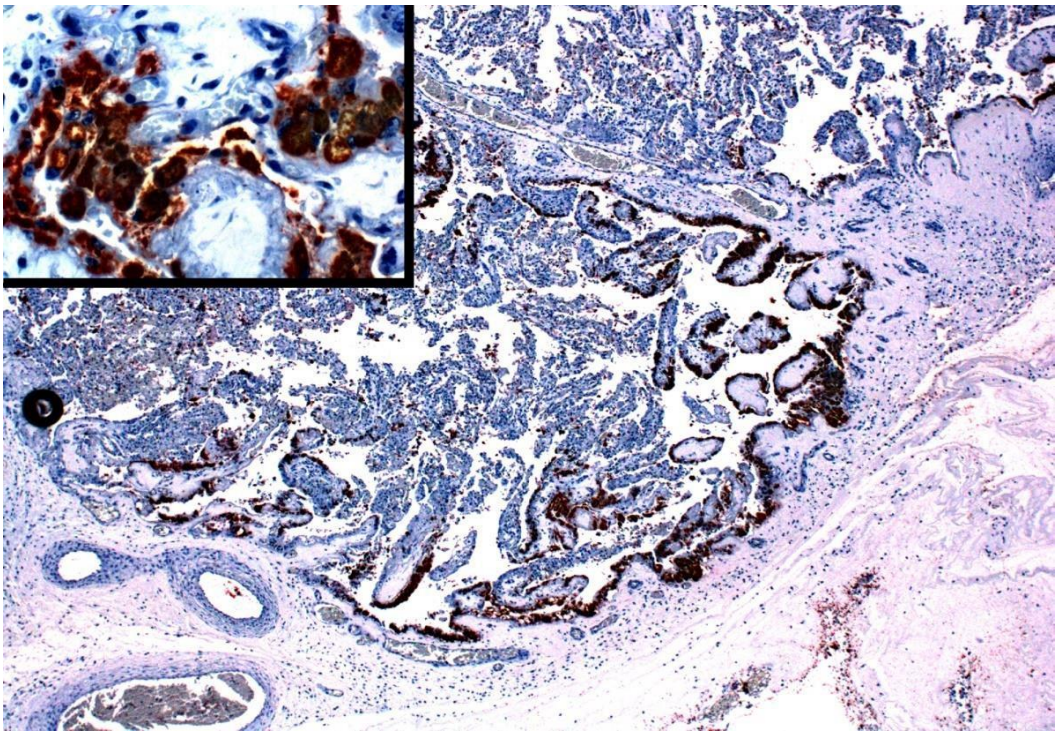


Figure 4. Cotyledonary placenta from aborted cow, case 4. Immunohistochemistry for *Coxiella burnetii* antigen, hematoxylin counterstain. There is diffuse intralesional immunoreactivity in the chorionic villi. Inset: higher magnification showing strong granular intracytoplasmic immunoreactivity in numerous trophoblasts.

DNA from three formalin-fixed, paraffin-embedded placentas (cases 1, 3 and 4) was extracted using the DNeasy Blood & Tissue Kit[a] (Qiagen, Hilden, Germany), concentrated using the DNA Clean & Concentrator™-5 Kit[b] (Zymo Research, Irvine, California) following the manufacturer's instructions and used as template for PCR. PCR was performed as previously described[29] in 25 µl final volume reactions, using a Green master mix[c] (Promega, Madison, Wisconsin), 800 nM of each primer (forward: TATGTATCCACCGTAGCCAGTC; reverse: CCCAACAACACCTCCTTATTC), and 5 µl template. An initial denaturation period of 5 minutes at 95ºwas followed by 40 cycles of denaturation at 94ºC (30 seconds), annealing at 62ºC (30 seconds) and extension at 72ºC (one minute). This was followed by a final extension step at 72ºC for three minutes. The PCR products were visualized on a 2% agarose gel. Purification of these reactions was achieved using a Quick Gel Extraction and PCR Purification Combo Kit[d] (ThermoFisher/Invitrogen, Waltham, Massachusetts). The gel extraction

products were sent to Genewiz[e] (South Plainfield, New Jersey) for Sanger sequencing, and sequences were aligned with SeqMan Pro 13[f] (DNASTAR, Lasergene 13, Madison, Wisconsin). *C. burnetii* DNA was amplified by PCR, and the amplicon sequenced in the three placentas examined (cases 1, 3 and 4).

Placenta, liver, lung, and abomasal fluid were spiked in blood and MacConkey agar and incubated at 37ºC in aerobic conditions. Same samples were spiked in Skirrow media at 37ºC in a micro-anaerobic condition with 5-10% of oxygen and 5-10% carbon dioxide, for four days.[14] Direct immunofluorescence (FA) for *Campylobacter* spp.[10] was done on impression smears from placenta, abomasal fluid, lung and liver, fixed with acetone at room temperature for 30 minutes, and incubated with the conjugate in a humid chamber at 37ºC for 30 minutes.[21] Placenta, liver, kidney and abomasal fluid were spiked in *Leptospira* Medium Base EMJH agar and incubated at 29ºC.[47] Placentas were analyzed under PCR for *Neospora caninum*. Immunohistochemistry for *Neospora caninum* was done in placentas and brains. The slides were immersed in citrate buffer and heat-induced antigen retrieval was performed in a decloaking chamber at 110°C for 30 min, after quenching the endogenous peroxidase with 3% hydrogen peroxide for 15 min. Goat polyclonal antibody against *Neospora caninum*[g] (VMRD, #210-70-NC, Pullman, WA) was applied as a primary antibody for 60 min, and anti-goat IgG horseradish peroxidase (HRP)-labeled polymer[h] (ImmPRESS/HRP Vector polymer enzyme detection kit; MP-7405, Burlingame, CA,USA) was used as the detection system (30 min incubation), with 3-amino-9-ethylcarbazole as the chromogen substrate solution[i] (DAKO #K3469, Santa Clara, CA USA). The positive control for the *Neospora caninum* IHC was a brain inoculated experimentally of a canine. The abomasal fluid was examined under dark field microscopy for *Campylobacter* spp and *Tritrichomonas foetus*. Pools of different parenchymatous organs and the four placentas were evaluated for Bovine Viral Diarrhea[6] and Parainfluenza 3 viruses.[30]

Sera from dams and fetal pericardial fluids were analyzed for the *Leptospira* serovars Grippotyphosa, Icterohaemorrhagiae, Pomona, Canicola, Hardjo Bovis, Hardjo Prajitno and Wolfii using microagglutination test (MAT).[47] All these analyses were negative and MAT was not reactive to the cutoff point (1/10). Commercial indirect ELISA[j] for specific IgG antibodies against *C. burnetii* (Q Fever Ab Test, IDEXX Laboratories, Westbrook, Maine) was performed in all cases and it was positive in

serum from dam in case 4. Samples were from maternal serum obtained either at the time of abortion or two to four months after the abortion.

Coxiella burnetii can be transmitted to the fetus either hematogenously (affecting mainly the liver) or transplacentary, causing pneumonia and lesions in the intestine due the consumption of the contaminated amniotic fluid.[3,13] The infected damn sheds large amounts of bacteria in the feces, milk and birth membranes, regardless the infection status of the birthed calf.[3]

Amongst post-natal animals, C. *burnetii* is transmitted by inhalation of aerosols from contaminated tissues shed the calving cows.[18,24] The placenta is the most frequent source of infection because the bacterial load can be as high as 10^9 cells per gram, and the infective dose can be as low as 1 cell.[25] It has been postulated that *C. burnetii* can also be transmitted by ticks.[20] Two microscopic forms of *C. burnetii* are recognized based on their pathogenicity, i.e. i) large cell variant and ii) small cell variant. Large cell variant is the vegetative form in infected cells. The small cell variant is the extracellular form, which is shed in milk, urine, feces, placenta and amniotic fluid.[44] The small cell variant is resistant to high temperature and desiccation, characteristics that confer this form of *C. burnetii* the capacity for airborne transmission and survive in the environment for years.[32]

In cattle, there is controversy about the role that *C. burnetii* may have in reproductive disorders other than abortion; such as infertility, premature delivery, endometritis, metritis and mastitis.[2,17] *C. burnetii*'s DNA is commonly detected in cases of endocarditis in bovines from slaughterhouses,[1] however the clinical significance of this finding is unknown. *Coxiella burnetii* DNA and its antigen have been detected in endometrial biopsies of cows with repeat breeding.[17] While these cows were not compared to healthy cows or with no history of repeated breeding, these findings suggest an association of *Coxiella burnetii* and history of reproductive disorder in bovines.[17] Differences in the clinical presentations of coxiellosis could be due differences in the bacterial genotype. Analysis of the genetic diversity of *C. burnetii* from different sources (bulk-tank milk and surface dust) by Multiple- Locus Variable number tandem repeats Analysis showed a high genotype diversity, but the presence in cattle of genotypes closely related to those identified in humans does not seem to be common.[37]

Coxiella burnetii has been considered an opportunistic pathogen associated with sporadic abortion in cattle.[3,40] However, an outbreak of Q fever in bovines in

Southeast Poland, affected 220 dairy cows, and at least 1,300 people.[8] While there was no abortion reported in this outbreak, seroconversions occurred in cows that had stillborn calves.[27,42] This is the largest outbreak in humans linked to bovines. In Netherlands there was also another significant epidemic episode of Q fever in people, but it was linked to a small ruminant source.[39]

The diagnosis of *C. burnetii* abortion in bovines is done by the detection of the agent in association with placental lesions.[3] Rarely Q fever produces abortion in cattle; only individual and sporadic cases of abortion have been reported.[3,4] In cattle, the shedding of the microorganism is higher during the peripartum period and can decrease to barely detectable levels after partum.[23] In cattle, *C. burnetii* can infect the mammary gland and lymph nodes, where it remains latent, but allows shedding of the bacteria during subsequent calving seasons and lactation.[32]

Microscopic examination of the placenta of aborted cattle coupled with histopathology[45] and IHC are key, since placentitis is the primary lesion caused by *C. burnetii,* with a strong statistical association between intralesional detection of the agent by IHC and placentitis.[9] In laboratory submissions that include the fetus but not the placenta, diagnosis of *Coxiella* abortion is challenging, since fetal lesions are usually absent or scarce.[9]When present, these are mainly restricted to the lungs.[9] Nevertheless fetal pneumonia is a non-specific lesion that frequently accompanies infectious placentitis of any cause. Even though molecular testing is adequate for rapid identification of *C. burnetii* in tissues and body fluids, the sole detection of this agent is of not diagnostic significance, since *C. burnetii* can be carried and excreted by healthy cattle.[3] Two of the aborted fetuses examined in this study had no gross or microscopic lesions, while the third fetus (case 3) showed only mild neutrophilic alveolitis with multinucleated syncytial cells. However, *C. burnetii* antigen was not detected by IHC associated with these lesions and their etiology remains, therefore, undetermined. In our four cases, the presumptive diagnosis of *C. burnetii* infection was based on the histologic lesions observed in the placenta and confirmed by IHC and PCR. These results are similar to those reported by Agerholm (2013) who described lesions in the placentas but not in the fetuses.[3]

Molecular detection by real-time PCR should be complemented by histopathologic analysis to improve the possibilities of arriving to an etiologic diagnosis of bovine abortion.[44] In a recent study from California, that evaluated 709 aborted bovine fetuses, *C. burnetii* was identified as the causal agent in only one (0.14%).[16] Similarly, an 11-

year study from Canada, revealed that only ten (1.4%) of 722 dairy cattle abortions were attributable to *C. burnetii*, and concluded that the agent was infrequently associated with bovine abortion.[9] Furthermore, in several case series from Argentina,[12,](Morrell E. Diagnostic characterization of the infectious causes of bovine abortion. [PhD dissertation]. La Plata, Argentina. Universidad Nacional de La Plata, Argentina. 2010), Brazil,[5] Uruguay (Easton C. Pathologic study of the main infectious causes of bovine abortion in Uruguay [master´s tesis]. Montevideo, Uruguay. Universidad de la República,2006; and the Unites States,[4,26] *C. burnetii* was not reported as a cause of bovine abortion. This could be due to a low frequency of infection and/or to the difficulties to reach an etiologic diagnosis of *C. burnetii* abortion.[16]

Several studies confirmed the presence of antibodies against *C. burnetii* in human and animal populations in South America indicating that the organism is present in the continent.[15,34] One study from Brazil established the presence of the agent in a human clinical case associated with PCR-positive animals.[31] In Uruguay, the presence of antibodies against *C. burnetii* in slaughterhouse workers has been associated with history of clinical signs, and a clinical case of endocarditis,[34] showing that this agent is an occupational hazard for veterinarians, farmers, laboratory technicians, and slaughterhouse personnel in Uruguay.

Healthy cows can carry and excrete *C. burnetii*[3] suggesting that in this cluster, carrier healthy cows might have been the source of infection. In this farm, before the onset of the disease, a herd of 77 Holstein cows of the New Zealand biotype had been introduced into the group. While it was not possible to conclude whether cattle movement was responsible for the introduction of *C. burnetii,* previous reports mentioned that dairy cow herds that purchase cows from abroad have 2.68 more chances of *C. burnetii* infection.[11] Nevertheless, the potential role of wildlife, other domestic animals such as dogs and cats, or people, in the transmission of the infection in this case cannot be ruled out.

Coxiella burnetii has been transmitted experimentally by several species of ticks, including *Rhipicephalus* spp. and *Ornithodoros* spp. ticks.[20] These two species of ticks are present in South America. *Coxiella burnetii* has been also found in *Ixodes* spp. ticks from Argentina,[36] and antibodies against this microorganism has been detected in dogs from the same country.[15] Even though these 3 genera of ticks are present in Uruguay,[35] they were not present in this dairy herd, as the dairy farm was in an official

free tick zone.[33]

The occurrence of bovine abortions by *C. burnetii* reported here, indicates that Q fever should be considered a potential hazard for dairy workers. In this cluster and following the Uruguayan Public Health Department guidelines, 27 farm and laboratory workers were examined for *C. burnetii* by an indirect fluorescent antibody test,[46] and ten (37%) of them had IgG Phase II and IgM phase II antibody titers against *C. burnetii* (data not shown). It is speculated that these individuals had contact with the aborted animals and fetal tissues. However, conclusions about the sources of their infection are difficult to make because the test used does not allow to determine duration of infection and the previous serologic status of these patients was unknown.[38,41]

It is concluded that *C. burnetii* may cause clusters of abortions in cattle, diagnostic tests for the identification of this agent should be included in the routine diagnostic panel of bovine abortion and the placenta is necessary for the diagnosis of *C. burnetii* abortion.

Acknowledgments for this chapter. The authors thank Bruno Lopez, Yisell Perdomo, Cecilia Monesiglio, Anderson Saravia and all graduate students from the animal health platform at INIA, Jennifer McKenna from UW, and Karen Sverlow and Juliann Beingesser from CAHFS for valuable technical assistance. In memory of Dr. Néstor D'Anatro from the "Division de Laboratorios Veterinarios" (DILAVE) of the Uruguayan "Ministerio de Ganadería, Agricultura y Pesca" (MGAP).

Sources and manufacturers

a DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany.

b DNA Clean & Concentrator™-5 Kit. Zymo Research, Irvine, California.

c Green master mix. Promega, Madison, Wisconsin.

d Quick Gel Extraction and PCR Purification Combo Kit. ThermoFisher/Invitrogen, Waltham, Massachusetts.

e Gel extraction products were sent to Genewiz. South Plainfield, New Jersey.

f SeqMan Pro 13. DNASTAR, Lasergene 13, Madison, Wisconsin.

g VMRD, Pullman, WA.

h ImmPRESS/HRP Vector polymer enzyme detection kit, Burlingame, CA, USA

i DAKO, Santa Clara, CA USA

j Q Fever Ab Test, IDEXX Laboratories, Westbrook, Maine.

Declaration of Conflicting Interest.-The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding.-This work was funded by grant FSSA_X_2014_1_105696 of the Uruguayan "Agencia Nacional de Investigación e Innovación" (ANII).

References.

1. Agerholm JS, et al. Presence of Coxiella burnetii DNA in inflamed bovine cardiac valves. BMC Vet Res 2016;13:69.
2. Agerholm JS. *Coxiella burnetii* associated reproductive disorders in domestic animals--a critical review. Acta Vet Scand 2013;55:13.
3. Agerholm JS. *Coxiella burnetii* and reproductive disorders in cattle. Infect Genet Evol 2014;28:150.
4. Anderson ML, et al. A survey of causes of bovine abortion occurring in the San Joaquin Valley, California. J Vet Diagn Invest 1990;4:283-287.
5. Antoniassi NAB, et al. Causas de aborto bovino diagnosticadas no Setor de Patologia Veterinária da UFRGS de 2003 a 2011. Pesq Vet Bras 2013;69:69-72.
6. Baszler, T. et al, Diagnosis of naturally occurring bovine Viral Diarrhea virus infection in ruminants using monoclonal antibody-based immunohistochemistry. Vet Pathol 1995;32:609-618
7. Berthová L, et al. The natural infection of birds and ticks feeding on birds with *Rickettsia* spp. and *Coxiella burnetii* in Slovakia. Exp Appl Acarol 2016;68:299-314.
8. Bielawska-Drózd A, et al. Q fever – selected issues. Annals of Agricultural and Environmental Medicine 2013;20:222-232.
9. Bildfell RJ, et al. *Coxiella burnetii* infection is associated with placentitis in cases of bovine abortion. J Vet Diagn Invest 2000;12:419-425.
10. Boenish, T. Handbook of Immunochemical Staining Methods. 3rd Ed. Dako Corporation, Carpinteria, California, 2001.
11. Boroduske A, et al. *Coxiella burnetii* (Q fever) infection in da1iry cattle and associated risk factors in Latvia. Epidemiol Infect 2017;10:2011-2019.
12. Campero CM , et al. Aetiology of bovine abortion in Argentina. Vet Res Commun 2003;27:359-369

13. *Campero CM, et al. Chapter 3. In:* Abortos y Otras Perdidas Reproductivas en Bovinos. Diagnóstico y Control. *1st ed*. Hemisferio Sur S. A. 2017:104-109.
14. Chaban B, García-Guerra A, et al. Isolation rates of *Campylobacter fetus* subsp. *venerealis* from bovine preputial samples via passive filtration on nonselective medium versus selective medium, with and without transport medium. Am J Vet Res 2013;74:1066-1069.
15. Cicuttin GL, et al. Serological evidence of Rickettsia and *Coxiella burnetii* in humans of Buenos Aires, Argentina. Comp Immunol Microbiol Infect Dis 2015;43:57-60.
16. Clothier K, Anderson M. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 2016;15:933-938.
17. De Biase D, Costagliola A, et al. Coxiella burnetii in Infertile Dairy Cattle With Chronic Endometritis. Vet Pathol 2018; 55:539-542
18. de Rooij MMT, et al. Detection of *Coxiella burnetii* in Ambient Air after a Large Q Fever Outbreak. PLoS One 2016;11:3.
19. Dilbeck PM, McElwain TF. Immunohistochemical detection of *Coxiella burnetii* in formalin-fixed placenta. J Vet Diagn Invest 1994;6:125-127.
20. Duron O, et al. The Importance of Ticks in Q Fever Transmission: What Has (and Has Not) Been Demonstrated? Trends Parasitol 2015;31:536-552.
21. Figueiredo JF, et al. Evaluation of direct fluorescent antibody test for the diagnosis of bovine genital campylobacteriosis. Rev Latinoam Microbiol 2002;44:118-123.
22. Giannitti F, et al. *Chlamydia pecorum*: fetal and placental lesions in sporadic caprine abortion. J Vet Diagn Invest 2016;28:184-189.
23. Hansen MS, et al. *Coxiella burnetii* associated placental lesions and infection level in parturient cows. Vet J 2011;190:135-139.
24. Jones RM, et al. The infectious dose of *Coxiella burnetii* (Q fever). Applied Biosafety 2006;1:32-41.
25. Kazar J. *Coxiella burnetii* infection. Ann N Y Acad Sc 2005;1063:105-114.
26. Kirkbride CA. Etiologic agents detected in a 10-year study of bovine abortions and stillbirths. J Vet Diagn Invest 1992;4:175-180.
27. Lapointe JM, et al. Placentitis due to *Coxiella burnetii* in a Pacific harbor seal (*Phoca vitulina richardsi*). J Vet Diagn Invest 1999;6:541-543.

28. Lorenz H, et al. PCR detection of *Coxiella burnetii* from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. Appl Environ Microbiol 1998;64:4234-4237.
29. Macías-Rioseco M, et al. Fetal Pathology in an Aborted Holstein Fetus Infected With Bovine Parainfluenza Virus-3 Genotype A. Vet Pathol 2018 Epub ahead of print
30. Mares-Guia MA, et al. Molecular identification of the agent of Q fever, *Coxiella burnetii* in domestic animals in State of Rio de Janeiro, Brazil. Rev Soc Bras Med Trop 2014;2:231-234.
31. Maurin M, Rauoult, D. Q Fever. Clin Microbiol Rev 1999;12:518-553.
32. Miraballes C, Riet-Correa F. A review of the history of research and control of *Rhipicephalus* (*Boophilus*) *microplus*, babesiosis and anaplasmosis in Uruguay. Exp Appl Acarol 2018;75(4):383-398.
33. Moreira RE, et al. Fiebre "Q" en Uruguay. Rev Inst Med trop S. Paulo 1987;29:168-173.
34. *Nava S, et al. Chapter 1. In:* Ticks of the Southern Cone of America. Diagnosis, Distribution, and Hosts with Taxonomy, Ecology and Sanitary Importance. *1st ed.* Elsevier, 2017:1-23.
35. Niemczuk K, et al. Q fever in a cattle herd and humans in the south-eastern Poland. Laboratory Diagnosis of the disease using serological and molecular methods. Bull Vet Inst Pulawy 2011;55:593-598.
36. Pacheco RC, et al. *Coxiella burnetii* in ticks, Argentina. Emerg Infect Dis 2013;19:344-3566.
37. Piñero A, et al. A Genetic diversity and variation over time of *Coxiella burnetii* genotypes in dairy cattle and the farm environment. Infect Genet Evol 2015;31:231-5.
38. Raven CF, et al. Solitary IgM phase II response has a limited predictive value in the diagnosis of acute Q fever. Epidemiol Infect 2012;140:1950-1954.
39. Roest HI, et al. The Q fever epidemic in The Netherlands: history, onset, response and reflection. Epidemiol Infect 2011;139:1-12.
40. Sawyer LA, et al. Q fever: current concepts. Reviews of Infectious Diseases 1987;9:935-946.
41. Schneeberger PM, et al. Real-time PCR with serum samples is indispensable for early diagnosis of acute Q fever. Clin Vaccine Immunol 2010;17:286-290.

42. Tylewska-Wierzbanowska S, et al. Epidemic of Q fever in Leszno district in Poland. Eur J Epidemiol 1991;7:307-309.
43. van der Hoek W, et al. Relation between Q fever notifications and *Coxiella burnetii* infections during the 2009 outbreak in The Netherlands. Euro Surveill 2012;17:20058.
44. van Schaik EJ, et al. Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii.* Nat Rev Microbiol 2013;11:561-573.
45. Vidal S, et al. Neglected zoonotic agents in cattle abortion: tackling the difficult to grow bacteria. BMC Vet Res 2017;13:373.
46. Wegdam-Blans MCA, et al. Evaluation of commonly used serological tests for detection of *Coxiella burnetii* antibodies in well-defined acute and follow-up sera. Clin Vaccine Immunol 2012;19:1110-1115.
47. Zarantonelli L, et al. Isolation of pathogenic Leptospira strains from naturally infected cattle in Uruguay reveals high serovar diversity, and uncovers a relevant risk for human leptospirosis. PLoS Negl Trop Dis 2018;12:9

CAPÍTULO 3:

Fetal pathology in an aborted Holstein fetus infected with bovine parainfluenza virus-3 genotype A (BPIV-3a, Respirovirus, Paramyxoviridae)

Melissa Macías-Rioseco, Santiago Mirazo, Francisco A. Uzal, Martín Fraga, Caroline Silveira, Leticia Maya, Franklin Riet-Correa, Juan Arbiza, Rodney Colina, Mark L. Anderson, Federico Giannitti

Plataforma de Investigación en Salud Animal, Instituto Nacional de Investigación Agropecuaria (INIA), La Estanzuela, Colonia, Uruguay (MMR, MF, CS, FRC, FG).
Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay (SM, JA).
California Animal Health Food Safety Laboratory System (CAHFS), University of California at Davis, CA, USA (FAU, MLA).
Laboratorio de Virología Molecular, Centro Universitario Regional (CENUR) Litoral Norte, Universidad de la República, Salto, Uruguay (LM, RC).
Veterinary Population Medicine Department, University of Minnesota, Saint Paul, MN, USA (FG).

Corresponding Authors: Federico Giannitti and Melissa Macías-Rioseco. Instituto Nacional de Investigación Agropecuaria (INIA), Ruta 50 km 11, La Estanzuela 70006, Colonia, Uruguay. Phone: +598 4574 8000, Fax: +598 4574 8012. Emails: fgiannitti@inia.org.uy (Giannitti), mmacias@inia.org.uy (Macías-Rioseco).

Vet Pathol. 24:300985818798117. doi: 10.1177/0300985818798117. [Epub ahead of print]

Abstract

The etiologic diagnosis of bovine abortion is complex. As a multifactorial syndrome potentially associated with many bacterial, viral, parasitic, toxic, metabolic, or genetic factors, the identification of the specific cause can be challenging, and the etiology remains undetermined in a large proportion of cases subjected to diagnostic investigation. Bovine parainfluenza virus-3 (BPIV-3) is a recognized respiratory pathogen of cattle. However, little is known of this agent as a reproductive pathogen. BPIV-3 has been identified in aborted bovine fetuses, although detailed descriptions of fetal pathology on natural cases are lacking in the scientific literature. This article describes and illustrates lesions in a fetus spontaneously aborted by a Holstein heifer, naturally infected with BPIV-3 genotype A, broadening the current knowledge on fetal pathology by this virus. In a diagnostic setting, identification of these lesions should encourage the pathologist to pursue specific BPIV-3 testing to increase the chances of reaching an etiologic diagnosis.

Keywords: Abortion; bovine parainfluenza virus-3; fetus; immunohistochemistry; reproductive pathology; *Respirovirus*; RT-PCR.

Bovine parainfluenza virus-3 (BPIV-3, genus *Respirovirus*, family *Paramyxoviridae*) was first isolated from the nasal discharge of cattle with respiratory disease in USA. It was named after its human counterpart, as it is antigenically and genetically related to human parainfluenza virus-3 (HPIV-3).[9] It has a single-stranded, negative-sense RNA genome, and a spherical to pleomorphic virion of 150-200 nm, comprising the nucleocapsid and lipid envelope. Three distinct genotypes, namely BPIV-3a through c, have been identified.[10,14]

BPIV-3 causes respiratory disease, and far less commonly, abortion. The virus spreads aerogenously, and even though infection is widespread in young dairy and beef cattle, spontaneous disease caused solely by BPIV-3 is rare.[4] However, the virus induces cytopathic effects on the mucociliary apparatus, bronchitis, bronchiolitis and alveolitis, with necrosis of the respiratory epithelium, and changes on the local and systemic inflammatory response that contribute to the establishment of bacterial infections, typical of enzootic pneumonia and bovine respiratory disease complex.[4,9] Information on BPIV-3 as an abortifacient is scant,[20] and descriptions of fetal lesions in spontaneously aborted fetuses in the scientific literature are succinct and limited to pulmonary lesions in one case.[1] Here we provide a detailed pathological description of pulmonary and enteric lesions in an aborted fetus naturally infected with BPIV-3a, broadening the current knowledge on fetal pathology caused by this agent.

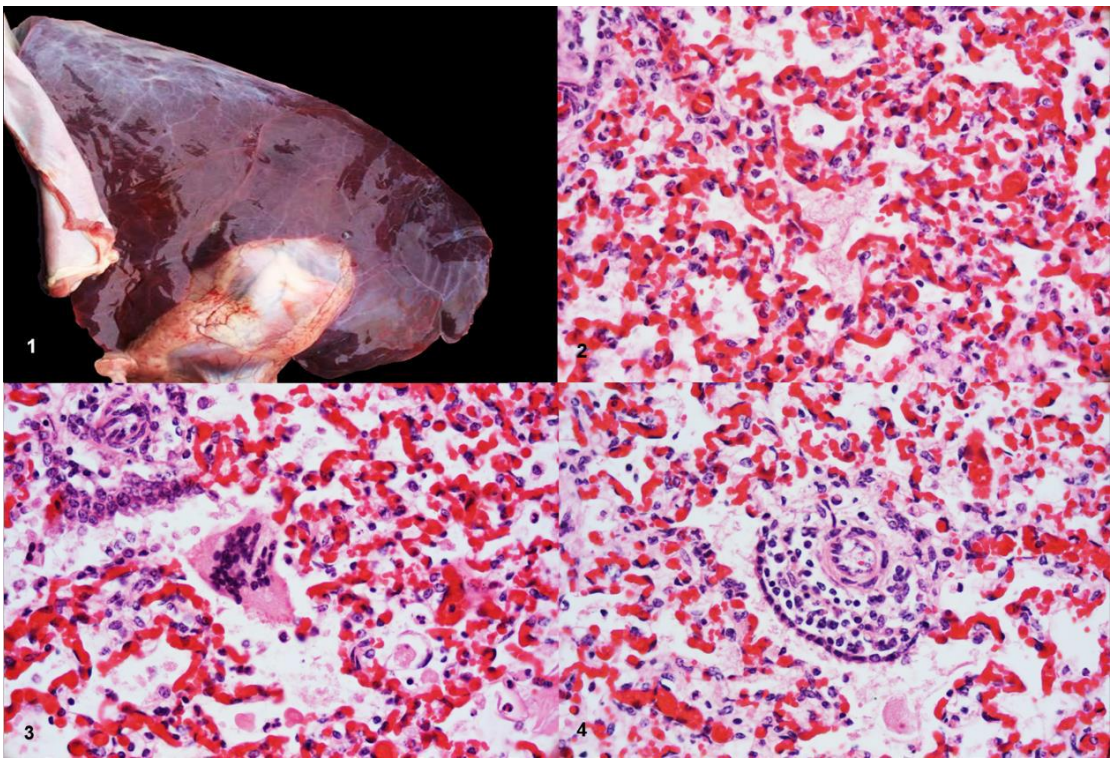
The case occurred on April-2017 in a herd of ~315 Holstein cattle at a *Brucella abortus*-free dairy farm in Uruguay. The aborted animal was a 25.5-month-old, first-calving heifer of unknown vaccination history that had been purchased pregnant, and introduced into the herd 48 days before. The heifer aborted a well preserved female fetus, 83 cm in crown-to-rump length consistent with 240-270 days gestation.[15]

The fetal autopsy revealed diffusely reddened, rubbery and unexpanded lungs (Fig.1). No other gross abnormalities were observed. The placenta was not available for examination. Tissues (lung, kidney, spleen, heart, adrenal gland, liver, skeletal muscle, trachea, tongue, brain, thymus, lymph node, abomasum, rumen, reticulum, small intestine, colon, and eyelid) were immersion-fixed in 10% neutral buffered formalin. Sections from all lung lobes sunk when immersed in formalin. Tissues were embedded in paraffin, sectioned at 4-5 µm, and stained with hematoxylin and eosin for histologic examination. Sections of lung and small intestine were processed by immunohistochemistry for the detection of BPIV-3 antigen. Slides were immersed in citrate buffer and heat-induced antigen retrieval was performed in a decloaking

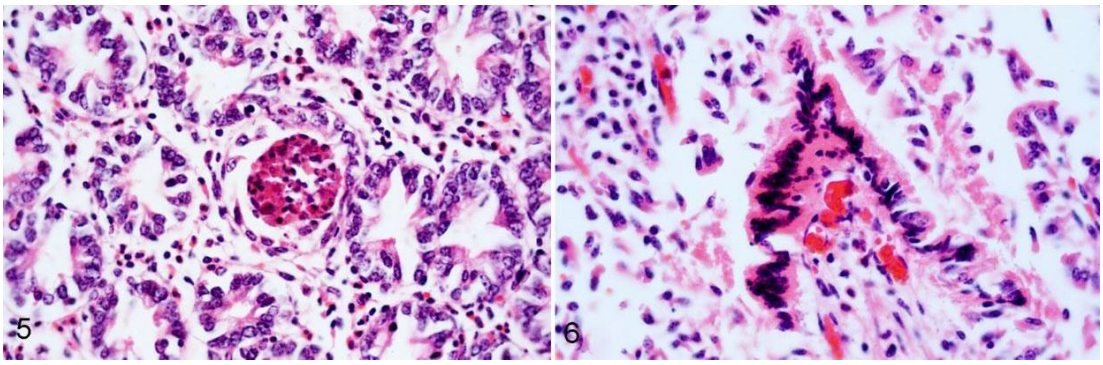
chamber at 121°C for 10 min, after quenching the endogenous peroxidase with 3% hydrogen peroxide for 10 min. Goat polyclonal antibody against BPIV-3 (VMRD, #210-70-PI3, Pullman, WA) was applied as a primary antibody for 15 min, and anti-goat horseradish peroxidase (HRP)-labeled polymer (Biocare, #GHP516, Pacheco, CA) was used as the detection system (15 min incubation), with 3-amino-9-ethylcarbazole as the chromogen substrate solution (ThermoScientific, #TA-125-SA, Fremont, CA).

Frozen lung was processed for BPIV-3 detection by reverse transcriptase (RT)-PCR.[10] Nucleic acid extraction was accomplished by TRIzol™ reagent (ThermoFisher, USA). RNA quality and integrity was assessed by NanoDrop™ (ThermoFisher, USA) and RT-PCR by amplifying a region within the beta actin gene.[18] BPIV-3 RT-PCR product was cloned into pJET1.2 Vector (ThermoFisher, USA) and 5 positive clones were sequenced. The plasmids containing the amplified region were purified and sequenced directly in both directions (Macrogen, Korea). Sequence analysis and phylogenetic reconstruction was performed with MEGA software. Supplementary Table 1 summarizes other ancillary test results.

Microscopically in the lungs, there was multifocal neutrophilic and histiocytic infiltrate in the alveolar spaces (alveolitis) with scattered intraluminal fibrin exudate and pyknotic or karyorrhectic cellular debris (necrosis), and a moderate number of syncytial cells with up to 40 nuclei within the alveolar and bronchiolar spaces (Figs. 2-3). The interstitium surrounding the epithelium of the terminal bronchioles and small-caliber arterioles and venules was multifocally expanded by moderate numbers of lymphocytes, macrophages and fewer plasmocytes, occasionally forming layers of up to 5 cells thick (peribronchiolitis with perivascular interstitial pneumonia) (Fig. 4). In the small intestine, there was scattered crypt dilation with epithelial necrotic debris filling the lumen (multifocal necrotizing cryptitis) with few neutrophils occasionally infiltrating the lamina propria (Fig. 5). Additionally, the superficial enterocytes occasionally formed syncytial cells that eventually sloughed into the intestinal lumen (Fig. 6).



Figures 1-4. Bovine parainfluenza virus-3 infection in an aborted Holstein fetus, lung. **Figure 1**. The pulmonary parenchyma is diffusely reddened and unexpanded, indicating lack of aeration. **Figure 2**. The alveolar spaces contain fibrin exudate, sloughed karyorrhectic epithelial cells and occasional alveolar macrophages. Hematoxylin and eosin (HE). **Figure 3**. There is a multinucleated epithelial syncytial cell in the lumen of a terminal bronchiole, with mild hyperplasia of the bronchiolar epithelium and mild peribronchiolar and periarteriolar interstitial inflammation. The adjacent alveolar spaces contain pyknotic epithelial cells, occasional neutrophils, macrophages and proteinic material. HE. **Figure 4**. The interstitium underlying a terminal bronchiole and adjacent arteriole is infiltrated by moderate numbers of lymphocytes, macrophages and plasma cells. There is proteinic material in the bronchiolar and alveolar spaces. HE.



Figures 5-6. Bovine parainfluenza virus-3 infection in an aborted Holstein fetus, small intestine. **Figure 5**. Focal necrotizing cryptitis with neutrophilic infiltrate in the adjacent lamina propria. HE. **Figure 6**. There is a multinucleated epithelial syncytial cell sloughed into the small intestinal lumen adjacent to the tips of intestinal villi. HE.

No lesions were found in the other tissues. No intracytoplasmic inclusions were identified. Immunohistochemistry revealed moderate widespread granular intracytoplasmic BPIV-3 antigen in bronchiolar epithelial cells, pneumocytes, syncytial cells, alveolar macrophages, and the alveolar and bronchiolar proteinic exudate (Fig. 7), as well as the necrotic crypts and syncytial enterocytes in the intestine. BPIV-3 genome was detected by RT-PCR. Sequences from the 5 clones were identical. Sequence analysis of the 274-bp matrix gene region confirmed the BPIV-3 identity and the strain, named LE017_1, was classified within the genotype A by phylogenetic reconstruction (Fig. 8). The sequence was deposited in GenBank (#MG976794).

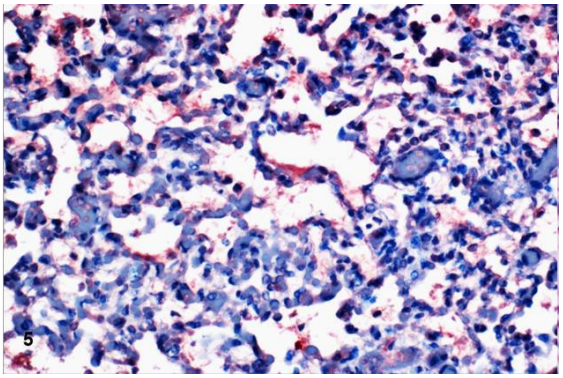


Figure 7. Bovine parainfluenza virus-3 infection in an aborted Holstein fetus, lung. Granular immunoreactivity in pneumocytes, cytoplasm of inflammatory/epithelial cells within alveolar spaces, and alveolar proteinic exudate. Immunohistochemistry for BPIV-3.

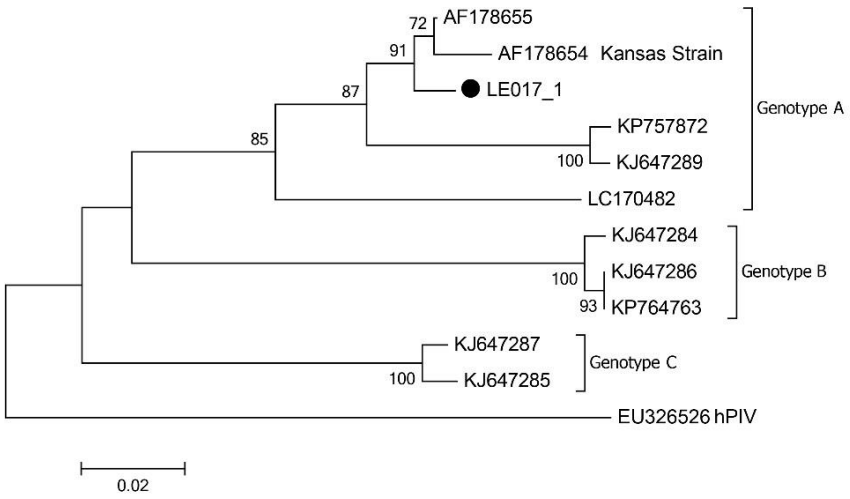


Figure 8. Phylogenetic tree based on a partial 274-bp region within the Matrix gene of BPIV-3. The tree was generated by using the neighbor-joining algorithm using Tamura-Nei as the best substitution model, as tested by the ModelTest v3.7 tool. The robustness of the tree was determined by bootstrap with 1000 replicates. Only values ≥70% are shown. BPIV-3 sequence obtained in this study (LE017_1) is highlighted (•)

The diagnosis of BPIV-3a abortion in this case was based on gross and microscopic findings in the fetus, coupled with intralesional detection of the agent by immunohistochemistry, and identification of the viral genome in the lung.[4] Other infectious abortifacients were ruled out by specific testing. The etiologic diagnosis of bovine abortion is complex. As a multifactorial syndrome potentially associated with many bacterial, viral, parasitic, toxic, metabolic, or genetic factors, identification of the specific cause can be challenging. Even at laboratories with experienced reproductive pathologists that routinely apply a broad panel of ancillary tests, most to detect infectious agents, an etiology remains undetermined in a large proportion of cases. A subset of these have inflammatory lesions suggesting an infection, although in many cases, failure of identifying a specific pathogen impedes reaching an etiologic diagnosis.[1,3,5,11]

Viral causes of bovine abortion are generally represented in low frequencies. An early study aiming at isolating viruses from aborted fetuses in Ohio identified infectious bovine rhinotracheitis virus (IBRV), and the first BPIV-3 fetal isolate.[16] No results of histopathologic examination were provided.

In a 10-year study that examined 8,962 abortions and stillbirths submitted to a

diagnostic laboratory in South Dakota, a viral etiology was determined in 948 (10.57%) cases. These included IBRV in 485 (5.41%) cases, and bovine viral diarrhea virus (BVDV) in 407 (4.54%) cases.[11] Other viruses included parvovirus, enterovirus (2 each), pseudorabies, adenovirus and BPIV-3 (one each).[12] These were considered miscellaneous infections, and fetal lesions were not described for the only case of BPIV-3 infection.

In a case series from California that investigated 468 bovine abortion cases in 1985-1989, viral causes of abortion were diagnosed in 26 (5.6%) cases, including 18 IBRV cases, 7 BVDV cases, and one BPIV-3 case.[1] A second series that included 709 cases of abortion submitted to the same laboratory in 2007-2012, again identified 25 (3.5%) and 12 (1.7%) cases of IBRV and BVDV infection, respectively, but no BPIV-3 cases were recorded.[5] Altogether, these works indicate that BPIV-3 is infrequently associated with bovine abortion in USA.

In case series from South America, including Uruguay,[8] Brazil,[6] and Argentina,[3] that collectively evaluated several hundred laboratory submissions, BPIV-3 was not reported as a cause of bovine abortion. However, BPIV-3a was isolated from an aborted bovine fetus in Brazil,[7] although no pathologic information was provided.

The pathogenesis of BPIV-3 as a respiratory pathogen in the postnatal life is relatively well understood;[4,9] however, the mechanisms associated with abortion are largely unknown. After aerogenous transmission, the hemagglutinin-neuraminidase glycoprotein in the viral envelope binds to sialic acid residues of glycoproteins and glycolipids of a variety of cell types in the respiratory tract, including tracheal epithelium, ciliated and non-ciliated bronchial and bronchiolar cells, and pneumocytes. The F glycoprotein mediates the fusion between the viral envelope and the host cell membrane, which allows the viral nucleocapsid to enter the cytoplasm. After uncoating of the matrix protein from the nucleocapsid, polymerase-mediated transcription of mRNA and translation of viral proteins take place in the cytoplasm of infected cells.[9] Several pathways may be involved in cellular damage; as a result, typical histologic lesions develop, particularly bronchiolitis. The epithelial cells of the small bronchioles may be rounded, swollen and vacuolated, with loss of cilia. The epithelium may be attenuated, and slough into the lumen. Eosinophilic intracytoplasmic inclusion bodies may be found in epithelial cells and alveolar macrophages, more commonly at 2-7 days post-infection. This is followed by epithelial proliferation beginning at ~14 days post-infection, type II pneumocyte

hyperplasia can occur, and epithelial syncytial cells may be present in the alveolar spaces, although this is more variable and may be isolate dependent. The airways may contain small numbers of neutrophils and macrophages, and the alveolar interstitium may be expanded by lymphocytes.[2,4,9]

Whether the same pathogenic mechanisms occur in the fetal life is unknown. Experimentally, in utero inoculation of bovine fetuses with a BPIV-3 strain isolated from an aborted fetus, produced both fetal lesions and immune response.[20] Neutralizing BPIV-3 antibody in pregnant heifers parenterally inoculated with BPIV-3 appears to prevent fetal infection.[20] However, viremia by BPIV-3 has been demonstrated and transplacental transmission seems plausible in naïve dams. It has been hypothesized that in areas where most of the adult cattle have antibodies against BPIV-3, this agent is not likely to be a major abortifacient; seronegative pregnant females might instead be susceptible to abortion.[20]

Necrotizing bronchiolitis, interstitial pneumonia and peribronchiolar lymphoid stimulation were demonstrated in surgically-removed fetuses inoculated in utero with BPIV-3 at mid-gestation.[19,20] Under these experimental conditions, some fetuses died and were aborted, while others survived the infection and were born weak, unable to rise, underweighted, or died in the perinatal period.[19,20] Descriptions of fetal lesions in spontaneous abortion cases are scarce and restricted to lymphocytic bronchointerstitial pneumonia in one case.[1] In the case presented here, the spectrum of pulmonary lesions included not only bronchointerstitial inflammation, but also necrosis, inflammatory and fibrinous exudate and epithelial syncytial cells in the bronchiolar/alveolar spaces. Although bronchial/alveolar syncytial cells are occasionally found in pneumonias induced by BPIV-3 postnatally,[4] to our knowledge this lesion has not been described in bovine fetuses. Interestingly, histological examination of the lung of a human fetus infected with HPIV-3, revealed multifocal pneumonia with necrosis, mononuclear and polymorphonuclear infiltrates, and "giant cells"[17] that might have represented epithelial syncytia. Additionally, the fetus in our case had multifocal necrotizing cryptitis and syncytial enterocytes in the small intestine, which also represent unprecedented lesions for this virus. In a diagnostic setting, identification of these lesions should encourage the pathologist to pursue specific BPIV-3 testing.

Current knowledge on BPIV-3 molecular epidemiology is somewhat limited. Sequencing analysis of BPIV-3 strains from USA revealed that, in addition to the BPIV-3a that had been previously identified in the country, there were two additional

genotypes circulating that, until then, had been described only in Australia (BPIV-3b) and Asia (BPIV-3c).[14] American genotypes B-C showed some divergence from the Australian and Asian strains, and cross-neutralization studies indicated that there were antigenic differences between these genotypes and the BPIV-3a included in commercial vaccines.[14] Information from South America is scant. In Argentina, BPIV-3a and BPIV-3c have been reported from cattle, and BPIV-3b was identified in water buffalo,[13] while BPIV-3a was identified in Brazil.[7] No information is available from Uruguay, since this represents the first scientific documentation of BPIV-3 in this country.

Acknowledgments for this chapter. The authors thank Bruno Lopez, Yisell Perdomo, Cecilia Monesiglio, Anderson Saravia and all graduate students from the animal health platform at INIA, Karen Sverlow and Juliann Beingesser from CAHFS for valuable technical assistance.

References

1. Anderson ML, Blanchard PC, Barr BC, Hoffman RL. A survey of causes of bovine abortion occurring in the San Joaquin Valley, California. *J Vet Diagn Invest.* 1990;2:283–287.
2. Campbell RS, Thompson H, Leighton E, Penny W. Pathogenesis of bovine parainfluenza 3 virus infection in organ cultures. *J Comp Pathol*. 1969;73:347–354.
3. Campero CM, Moore DP, Odeón AC, Cipolla AL, Odriozola E. Aetiology of bovine abortion in Argentina. *Vet Res Commun*. 2003;27:359–369.
4. Caswell JL, Williams KJ. Respiratory system. In: Maxie MG, ed. *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*. 6th ed. Vol. 2. St. Louis, MO: Elsevier; 2016:541.
5. Clothier K, Anderson M. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. *Theriogenology*. 2016;85:933–938.
6. Corbellini LG, Pescador CA, Frantz F, et al. Diagnostic survey of bovine abortion with special reference to *Neospora caninum* infection: Importance, repeated abortion and concurrent infection in aborted fetuses in Southern Brazil. *Vet J* 2006;172:114–120.

7. de Almeida Vaucher R, Dezen D, Simonetti AB, Spilki FR, Roehe PM. Phylogenetic characterization of bovine parainfluenza 3 from contaminated cell cultures and field isolates from Brazil. *Braz J Microbiol*. 2011;42:1440–1444.
8. Easton C. Estudio patológico de las principales causas infecciosas en el aborto bovino en Uruguay. Master's thesis, Universidad de la República, Montevideo, Uruguay, 2006. In Spanish.
9. Ellis JA. Bovine parainfluenza-3 virus. *Vet Clin North Am Food Anim Pract*. 2010;26:575–593.
10. Horwood PF, Gravel JL, Mahony TJ. Identification of two distinct bovine parainfluenza virus type 3 genotypes. *J Gen Virol*. 2008;89:1643–1648.
11. Kirkbride CA. Etiologic agents detected in a 10-year study of bovine abortions and stillbirths. *J Vet Diagn Invest*. 1992a;4:175–180.
12. Kirkbride CA. Viral agents and associated lesions detected in a 10-year study of bovine abortions and stillbirths. *J Vet Diagn Invest*. 1992b;4:374–379.
13. Maidana SS, Lomonaco PM, Combessies G, et al. Isolation and characterization of bovine parainfluenza virus type 3 from water buffaloes (*Bubalus bubalis*) in Argentina. *BMC Vet Res*. 2012;8:83.
14. Neill JD, Ridpath JF, Valayudhan BT. Identification and genome characterization of genotype B and genotype C bovine parainfluenza type 3 viruses isolated in the United States. *BMC Vet Res*. 2015;11:112.
15. Roberts SJ. *Veterinary obstetrics and genital diseases (Theriogenology)*. Woodstock, VT: David & Charles Inc., 1986:92.
16. Sattar SA, Bohl EH, Senturk M. Viral causes of bovine abortion in Ohio. *J Am Vet Med Assoc*. 1965;147:1207–1210.
17. Seidman DS, Nass D, Mendelson E, Shehtman I, Mashiach S, Achiron R. Prenatal ultrasonographic diagnosis of fetal hydrocephalus due to infection with parainfluenza virus type 3. *Ultrasound Obstet Gynecol*. 1996;52–54.
18. Silva-Benítez E, Soto-Sáinz E, Pozos-Guillen A, et al. Quantification of DNA in urinary porcine bladder matrix using the ACTB gene. *In Vitro Cell Dev Biol Anim*. 2015;51:1040–1046.
19. Swift BL. Bovine parainfluenza-3 virus experimental fetal disease. *J Am Vet Med Assoc*. 1973;163:861–862.
20. Swift BL, Trueblood MS. The present status of the role of the Parainfluenza-3 (PI-3) virus in fetal disease of cattle and sheep. *Theriogenology*. 1974;2:101–107.

CAPÍTULO 4:

Abortion outbreak caused by *Campylobacter fetus* subspecies *venerealis* and *Neospora caninum* in a bovine dairy herd

Melissa Macías-Rioseco1, Rubén D. Caffarena1,2, Martín Fraga1, Caroline Silveira1, Federico Giannitti1,3, Germán Cantón4, Yanina P. Hecker5, Alejandra Suanes6, Franklin Riet-Correa1*

Correspondence to: F. Riet-Correa (e-mail: frcorrea@inia.org.uy)

1Plataforma de Investigación en Salud Animal, Instituto Nacional de Investigación Agropecuaria (INIA), Ruta 50 km 11, La Estanzuela, Uruguay. CP 70000. 2 Universidad de la República, Facultad de Veterinaria, Montevideo, Uruguay. 3 University of Minnesota, Veterinary Population Medicine Department, MN, USA. 4Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina. 5Consejo Nacional de Investigaciones Científicas y Técnicas, Balcarce, Argentina. 6Dirección de Laboratorios Veterinarios, Ministerio de Ganadería Agricultura y Pesca, Montevideo, Uruguay.

Revista Mexicana de Ciencias Pecuarias. Accepted. 5008-11108-1

Abstract

In November 2015, an abortion outbreak occurred in a commercial dairy herd of 650 Holstein cows in Florida department, Uruguay. Forty-five cows aborted within three weeks. Five fetuses were subjected to gross and microscopic pathologic examination, and microbiological testing. One fetus had fibrinous epicarditis and peritonitis, and neutrophilic bronchopneumonia. *Campylobacter fetus* subsp. *venerealis* was detected by direct immunofluorescence, isolated and identified by PCR and sequencing of the 16S rDNA in the abomasal fluid and/or lung. Histologic examination of two other fetuses revealed non-suppurative necrotizing encephalitis, lymphohistiocytic myositis and myocarditis, and lymphocytic interstitial nephritis. In these fetuses, *N. caninum* antigen was detected intralesionally by immunohistochemistry, and *N. caninum* DNA was amplified by PCR on formalin-fixed paraffin-embedded brain. Antibodies against *N. caninum* were detected by indirect immunofluorescence in 10 of 27 cows, with titers ranging from 1/200 to 1/3200. The results indicate that two abortigenic microorganisms may coexist and cause contemporaneous abortion in a herd. We highlight the importance of performing multiple diagnostic tests in various aborted dams and fetuses from the same herd for the etiologic confirmation of bovine abortion syndrome.

Keywords: bovine abortion, *Campylobacter fetus* subsp. *venerealis*, *Neospora caninum*, diagnosis of abortion

Campylobacter fetus subspecies *venerealis* is the causal agent of bovine genital campylobacteriosis.[1] Bulls can carry the bacterium asymptomatically in the prepuce for indefinite time and transmit the agent to females at mating. Infected females can develop infertility, embryonic death or abortion. Abortion can occur at any gestational age, but it is more commonly diagnosed in the fourth to sixth month of gestation.[2] Lesions caused by *C. fetus venerealis* include endometritis, placentitis, fetal serositis, hepatitis and pneumonia.[1]

The protozoan *Neospora caninum* is an important cause of abortion in beef and dairy cattle in South America.[3] Members of the *Canidae* family are definitive hosts and shed oocysts in feces.[4] Cattle are intermediate hosts and get infected with *N. caninum* by ingestion of oocysts or by transplacental transmission. The definitive hosts acquire the infection by ingesting bradyzoites that are encysted in the tissues of the intermediate hosts. Depending on the gestational age at the time of infection, fetal death with either abortion or mummification can occur. If the infection takes place within the first 100 days of gestation, the chances of fetal survival are low because there is incomplete development of the fetal immune system.[4] Abortion due to *N. caninum* frequently occurs during the second or third trimester. If the fetus develops an immune reaction against *N. caninum*, it is born as seropositive calf. However, the birth of seronegative calves from seropositive dams can occasionally occur[4]. Necropsy findings in aborted fetuses are scant, fetuses can be severely autolytic or mummified. Grossly, the placenta can show necrosis of the cotyledons with no changes of the intercotyledonary region. The fetal heart and skeletal muscles can have gray to whitish foci, that microscopically are characterized by necrosis and inflammation. The main microscopic lesions in the fetus are multifocal non-suppurative necrotizing encephalitis with gliosis, myocarditis and myositis, which are highly specific of this protozoon.[4]

In this report we describe a bovine abortion outbreak in a commercial dairy farm caused by the concurrent action of two different pathogens. The importance of performing multiple diagnostic tests in various fetuses and serological studies in the cows is highlighted.

The outbreak occurred in a Brucellosis free dairy herd with 650 milking Holstein cows in a semi-extensive system with periods of confinement of variable lengths depending on pasture availability. The farm was located in Florida department, Uruguay. The average daily milk production was approximately 20L /cow. Calving was scheduled

in autumn-winter, and artificial insemination was performed from May to October, followed by natural breeding with bulls. The affected farm worked with a second dairy farm where they received cows for insemination that have calved at least two months before. After being inseminated, these cows remained in the farm during lactation. A total of 45 cows aborted during a period of three weeks in November 2015. Five fetuses (cases 1-5) were necropsied, the gestational age was estimated based on the crown-to-rump length and other gross characteristics of the fetuses.[5] There was no placenta submitted for examination in any of the cases. For histology, fetal tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-6 µm, and stained with hematoxylin and eosin. Immunohistochemistry (IHC) was performed in sections of brain for *N. caninum*, sections of kidney and liver for *Leptospira* spp., and in liver, heart, and lung for bovine viral diarrhea virus (BVDV).[6-8] Titers of antibodies against *Leptospira* spp. were determined by microagglutination test (MAT) on samples of pericardial/thoracic fluid from the five fetuses, with a cutoff point $\geq$1/10. Samples of abomasal fluid, and liver from the five aborted fetuses were spiked in blood Agar in microaerophilic conditions.

The molecular identification of *N. caninum* was done from formalin-fixed paraffin-embedded sections of brain in those fetuses with microscopic brain lesions typical of neosporosis. The DNA was isolated using a commercially available kit (DNeasy Tissue Kit, QIAGEN Group, Germany) according to the manufacturer's recommendations, and DNA concentration was measured using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® Instruments, Inc., Vermont, USA). *Neospora caninum* DNA was assessed by a nested-PCR targeting the internal transcribed spacer one (ITS1) region with four oligonucleotides, as previously described.[9]

The diagnosis of *Campylobacter* infection was done by bacterial culture, inoculating samples of fetal abomasal fluid, lung, and liver in Skirrow agar. Incubation was performed for 48 h at 37ºC in an AnaeroJar™ (Oxoid) with a microaerobic environment (approximately 5-10%O_2, 5-10% CO_2) generated with CampyGen™ sachets (Oxoid).[10] Direct immunofluorescence was done on smears of fetal abomasal fluid (20 µL) fixed in acetone at 20^0C for 30 minutes, using a commercially available fluorescein-isothiocyanate conjugated antiserum (FITC) against *Campylobacter fetus* (Biotandil, Argentina), with appropriate positive and negative controls provided with

the kit. Incubation was performed inside a humid chamber at 37ºC for 30 minutes, slides were then visualized under an AXI0 Lab A.1 microscope with a FITC filter and 470 nm UV light.

For the molecular identification of the isolates, DNA was extracted using the Gene bacterial genomic DNA extraction kit (Sigma-Aldrich, USA), following by two separate multiplex PCR protocols that amplify specific regions of the *C. fetus* genome that discriminate between *C. fetus* subspecies.[11,12] Additionally, the almost complete gen that codifies the 16S rRNA was amplified using the universal primers 27F and 1492R[13]. The PCR products were purified and sequenced at Macrogen Inc., Seoul, South Korea. The obtained sequences were compared with sequences from public databases using the "Classifier" tool from the Ribosomal Database Project and BLASTn from the National Center for Biotechnology Information[14,15]. Indirect fluorescent antibody test for the detection of anti-*Neospora caninum* antibodies was done in serum of 27 cows from the affected herd at the Division of Veterinary Laboratory of the Uruguayan Ministry of Agriculture, Livestock and Fishery, following their standard protocols.

All five necropsied fetuses (cases 1-5) had approximate gestational ages of 180 days. Grossly, case 1 had diffuse fibrinous epicarditis (Fig. 1) and peritonitis. Histologically in this fetus, there was neutrophilic bronchopneumonia and epicarditis. *Campylobacter fetus* was detected by direct immunofluorescence and isolated from abomasal fluid and lung. Identification of the isolate was further confirmed by PCR, which yielded amplification products of sizes corresponding to those described for C. *fetus* subsp. *venerealis*[11,12]. Additionally, the 16S rDNA gene sequence of the isolates was compatible with *C. fetus.* No *Campylobacter* spp. were isolated from samples of cases 2-5.

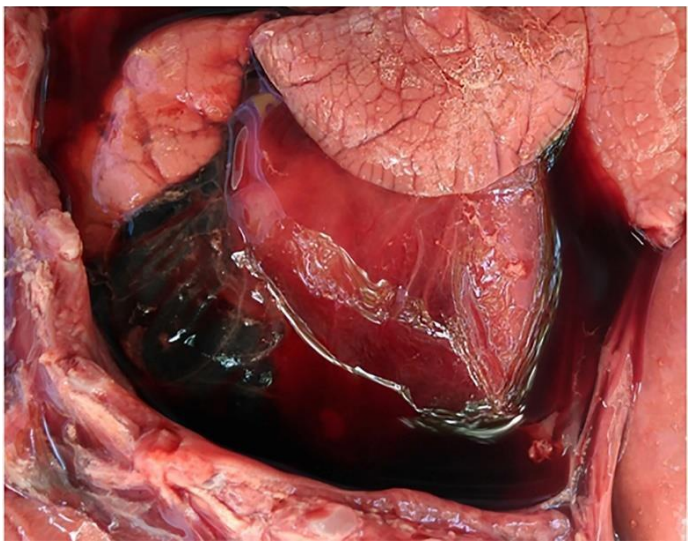


Figure 1. Case1, aborted by *Campylobacter fetus* subsp. *venerealis*. The epicardium is covered by moderate to large amount of fibrinous material along with serosanguinous fluid.

No significant gross lesions were observed in cases 2-5, and no microscopic lesions were observed in cases 2-3. However, in cases 4 and 5, histology revealed multifocal necrotizing non-suppurative encephalitis (Fig. 2), lymphocytic and histiocytic myocarditis and myositis, and lymphocytic interstitial nephritis. *Neospora caninum* antigen was detected intralesionally in the brain of these two fetuses by IHC (Fig. 2 inset) antibody titers to *N. caninum* ranged from 1/200 to 1/3200 in 10 of the 27 examined cows. Additionally, PCR for *N. caninum* DNA was positive in both cases. BVDV IHC was negative in liver, heart, and lung in cases 2, 4 and 5. Lastly, the IHC for *Leptospira* spp. was negative in kidney and liver from all fetuses. No antibody titers against *Leptospira* spp. were detected in any of the five fetuses. Other abortigenic pathogens were ruled out, such as *Brucella* spp. Their negativity was based on negative isolation of the pathogen and the absence of the pathogen in association with the compatible lesions.

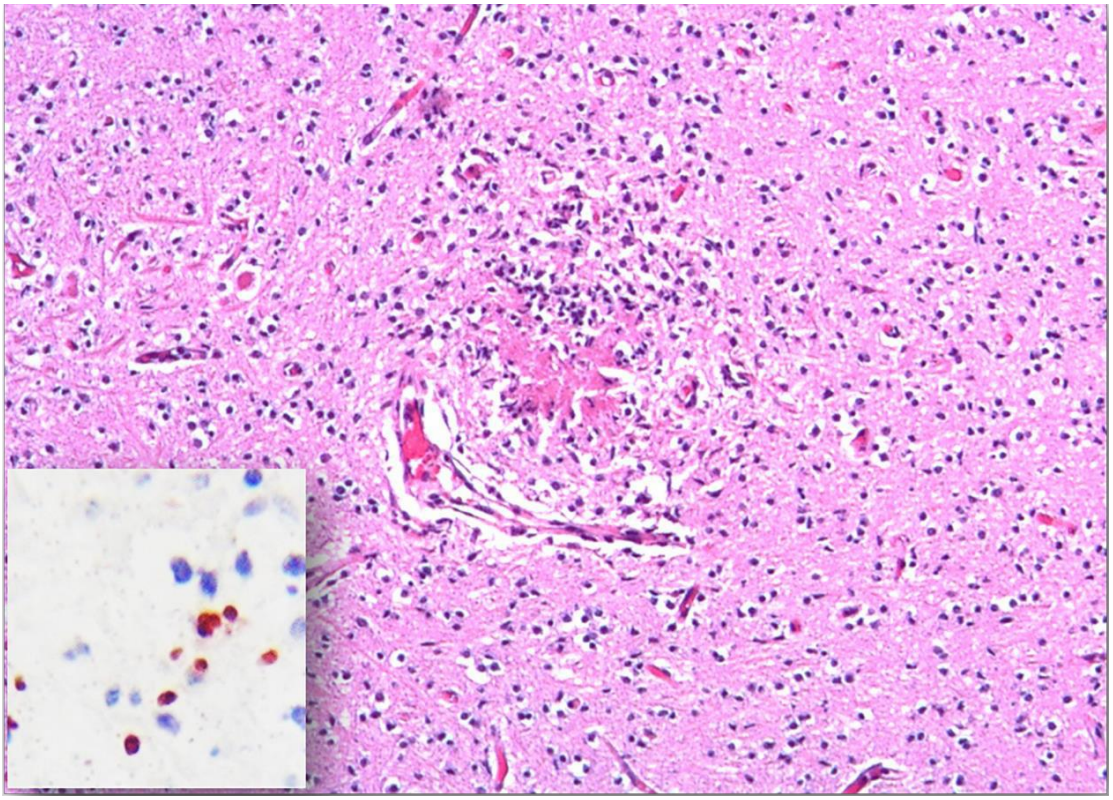


Figure 2. Fetal brain from case 4 aborted by *Neospora caninum.* The neuropil is disrupted by necrotizing non-suppurative multifocal encephalitis. Hematoxylin and eosin stain. **Inset.** There is immunoreaction against *Neospora caninum* antigen within the affected sections of brain.

The etiologic diagnosis of bovine abortion is complex because multiple potential causes can be involved, and fetal autolysis can preclude the identification of the etiologic agent. In this outbreak, the identification of two abortigenic pathogens in 3/5 fetuses suggests that the examination of various fetuses is recommended. While coinfection by multiple abortifacients has been reported,[16,17] little has been discussed about the concurrent detection of different pathogens in different aborted fetuses and in outbreaks of abortion in dairy farms. Abortion outbreaks can be caused by different infectious agents contemporaneously.

The main identified causes of bovine abortion in dairy and beef cattle in South America are infectious.[18-22] In one study from Uruguay, the most frequent cause of bovine abortion identified in laboratory submissions was leptospirosis (41% of 241 cases with diagnosis), followed by neosporosis (36%) and campylobacteriosis (12%)[20]. In Argentina, leptospirosis was the third most frequently detected cause (7.3%)[21] while in Brazil it was diagnosed in 0.6% of the abortions[22]. Such differences may be due to

the different frequencies of leptospirosis in these countries, but also to the different laboratory techniques used for the diagnosis and interpretation of the results. The diagnosis in the Uruguayan study was based on the presence of high titers of antibodies in aborted cows (≥1/800) and/or fetuses (≥1/10), whereas in Argentina and Brazil the etiologic diagnosis was based on detection of *Leptospira* spp. by PCR, immunofluorescence, immunohistochemistry, and/or Warthin Starry stain in fetal samples.[19,22]

Tests that aim at detecting the agent in the aborted fetuses are more suitable for the confirmatory diagnosis of abortion by *Leptospira* spp. than serologic tests performed on the dam´s serum of fetal fluids. The microbiologic and pathologic evaluation of the placenta in cases of abortion is key to increase the chances of reaching a diagnosis. For some diseases, such as coxiellosis or chlamydiosis, it is difficult to arrive to an etiologic diagnosis if the placenta of aborted cows is not evaluated. In this outbreak, no placentas were examined, and this could have been a limitation for the determination of the diagnosis in two of the five fetuses. Previous reports in the USA show that placentitis can result in abortion, in the absence of fetal lesions.[23]

In this report, the diagnosis of *C. fetus* subsp. *venerealis* was confirmed in one of the fetuses. The affected herd practiced artificial insemination followed by natural breeding. A national survey including 340 farmers indicated that only 21% of the dairy farms used artificial insemination and 29% used natural breeding after artificial insemination. Half of the farms used only natural breeding[22]. These data suggest that bovine campylobacteriosis, diagnosed for the first time in Uruguay in 1970 in dairy cows[25], is still a health problem in dairy farms in the country. Nevertheless, natural breeding maintains the risk of bovine genital campylobacteriosis and should be avoided when possible. Neosporosis was diagnosed in two fetuses and about 37% of the examined cows had titers against *N. caninum*. A serologic survey done in beef cattle in Uruguay showed that in 2006, neosporosis was present in 69.2% of 229 farms, and that 14.3% of the cows and 12.9% of the heifers were seropositive[26] proving that *N. caninum* is endemic in the Uruguayan bovine population, including dairy cattle.[27]

In this outbreak, antibody titers against *L. interrogans* serovars were detected in serum of 15/18 cows examined by MAT (data not shown). Unfortunately, the vaccination status of the herd unknown, and whether these dams had aborted or not was not recorded. The antibodies detected were against serovars Pomona (13 cows), Hardjo-

prajitno (nine cows), Wolfii (nine cows) and Hadjo-bovis (seven cows), with titers ranging from 1/200 to 1/3200. In the absence of fetal lesions compatible with leptospirosis coupled with negative IHC results, and the negative MAT results in the thoracic/cavitary fetal fluids, we conclude that none of the five examined fetuses were infected with *Leptospira* spp.

Abortion outbreaks can be caused by different infectious agents contemporaneously in the same herd. In such cases, it is necessary to perform necropsies in many fetuses using the specific techniques for each agent and, if possible, to evaluate the placenta, along with the serum of the dams.

Acknowledgments for this chapter.-The authors thank Cecilia Monesiglio, Anderson Saravia, Yisell Perdomo and all graduate students of the animal health platform at INIA. This work was funded by grant FSSA_X_2014_1_105696 of the Uruguayan "Agencia Nacional de Investigación e Innovación" (ANII).

References.

1. Michi AN, Favetto PH, Kastelic J, Cobo ER. A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. Theriogenology 2016;85(5):781-791.
2. de Vargas AC, Costa MM, Vainstein MH, Kreutz LC, Neves JP. *Campylobacter fetus* subspecies *venerealis* surface array protein from bovine isolates in Brazil. Curr Microbiol 2002;45(2):111-114.
3. Moore DP. Neosporosis in South America. Vet Parasitol 2005;127(2):87-97
4. Donahoe SL, Lindsay SA, Krockenberger M, Phalen D, Šlapeta J. A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. Int J Parasitol Parasites Wildlife 2015;24(2):216-238
5. Roberts SJ. Veterinary obstetrics and genital diseases (Theriogenology). Woodstock, VT: David & Charles Inc., 1986:92
6. Barr B, Rowe JD, Sverlow KW, BonDurant RH, Ardans AA, Oliver MN et al. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. J Vet Diagn Invest 1994;6(2):207-15.
7. Baszler TV, Evermann JF, Kaylor PS, Byington TC, Dilbeck PM. Diagnosis of naturally occurring bovine viral diarrhea virus infections in ruminants using

monoclonal antibody-based immunohistochemistry. Vet Pathol 1995;32(6):609-18.

8. Szeredi L, Haake DA. Immunohistochemical identification and pathologic findings in natural cases of equine abortion caused by leptospiral infection. Vet Pathol 2006;43(5):755–761.
9. Buxton D, Maley SW, Wright S, Thomson KM, Rae AG, Innes EA. The pathogenesis of experimental neosporosis in pregnant sheep. J Comp Pathol 1998;118(4):267-79.
10. OIE. Bovine genital campylobacteriosis (Chap. 2.4.4) (2017). Available from: http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.04_BGC.pdf. Accessed: July 12, 2018.
11. Hum S, Quinn K, Brunner J, On SL. Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. Aust Vet J 1997;(75): 827-831.
12. Iraola G, Hernández M, Calleros L, Paolicchi F, Silveyra S, Velilla A, et al. Application of a multiplex PCR assay for *Campylobacter fetus* detection and subspecies differentiation in uncultured samples of aborted bovine fetuses. J Vet Sci 2012;(13):371-376.
13. Linton D, Owen R, Stanley J. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. Res Microbiol 1996;(147):707-718.
14. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian Classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;(73):5261-5267.
15. Altschul S, Madden T, Schäfer A, Zhang J, Miller W, Lipman D. Grapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;(25):3389-3402.
16. Björkman C, Alenius S, Manuelsson U, Uggla A. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. Vet J 2000;159(2):201-6.
17. Quinn HE, Windsor PA, Kirkland PD, Ellis JT. An outbreak of abortion in a dairy herd associated with *Neospora caninum* and bovine pestivirus infection. Aust Vet J 2004;82(1-2):99-101.

18. Bove R, López F, Perera C, Carracelas B, Torres-Dini D, De Souza G, et al. Diagnóstico de Campylobacter fetus venerealis por PCR, en un aborto bovino espontáneo. Vete Montevideo 2013; 49 (192):20-28
19. Campero CM, Moore DP, Odeón AC, Cipolla AL, Odriozola E. Aetiology of bovine abortion in Argentina. Vet Res Commun 2003;27(5):359-369
20. Easton C. Estudio patológico de las principales causas infecciosas en el aborto bovino en Uruguay [Master´s thesis]. Montevideo, Uruguay: Universidad de la República; 2006
21. Morrell E. Caracterización diagnóstica de las causas infecciosas del aborto bovino [PhD Thesis]. Ciudad de la Plata, Argentina: Universidad Nacional de La Plata; 2010
22. Antoniassi NAB, Juffo GD, Santos AS, Pescador CA, Corbellini, LG, Driemeier D. Causas de aborto bovino diagnosticadas no Setor de Patologia Veterinária da UFRGS de 2003 a 2011. Pesquisa Veterinária Brasileira 2013;33(2):155-160
23. Clothier K, Anderson M. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 2016;85(5):933-938
24. Instituto Nacional de Investigación Agropecuaria. Encuesta Lechera INALE 2014. http://www.inale.org/innovaportal/file/4086/1/encuesta-lechera-2014--presentacion-resultados-preliminares-foro.pdf. Accessed: July 12, 2018.
25. Stella JL, Canabez, F. El diagnóstico de la vibriosis genital de los bovinos del Uruguay [resumen]. Congreso Latinoamericano de Microbiología. Punta del Este, Uruguay. 1971.
26. Banales P, Fernandez L, Repiso M, Gil A, Dargatz D, Osawa T. A nationwide survey on seroprevalence of *Neospora caninum* infection in beef cattle in Uruguay. Vet Parasitol 2006;139(1-3):15-20
27. Kashiwazaki Y, Gianneechini RE, Lust M, Gil J. Seroepidemiology of neosporosis in dairy cattle in Uruguay. Vet Parasitol 2004;120(1-2):139-44

7. CONCLUSIONES O CONSIDERACIONES FINALES

Las tres causas más frecuentes de aborto en ganado lechero de esta serie de casos fueron *Neospora caninum, Coxiella burnetii* y *Campylobacter fetus* subsp *venerealis. Neospora caninum* y *Campylobacter fetus* subsp *venerealis* ya eran conocidos como causa de abortos en el Uruguay, pero *C. burnetii* fue diagnosticada por la primera vez como causa de abortos en el País, lo que representa una preocupación, también, para la salud pública. Este documento identificó la frecuencia de los diferentes patógenos, observándose agentes causando brotes de abortos y también abortos esporádicos. Semejante a lo observado en otros trabajos, la mayoría de los abortos son de causa indeterminada, pero siempre es importante buscar la causa de aborto, analizando fetos y placentas. A pesar de tener un historial de las enfermedades reproductivas que causan aborto en el rodeo, es sustancial diagnosticar las causas de abortos, pues varios agentes distintos de forma simultánea pueden causar brotes de abortos o abortos ocasionales. Es importante la estandarización de pruebas diagnósticas, para aumentar las posibilidades de lograr un diagnóstico. En este trabajo se identificaron algunas causas de aborto que anteriormente no habían sido descriptas en el Uruguay: *Coxiella burnetii*, el virus Parainfluenza-3 bovino, *Salmonella enterica* serovar Newport. En el futuro será necesario buscar otras posibles causas de aborto como *Mycoplasma* spp y *Chlamydophila* spp que también pueden estar causando abortos en el Uruguay.

Para el diagnóstico de aborto, particularmente por agentes infecciosos, como *Coxiella burnetii*, es fundamental, además de examinar el feto, realizar análisis patológicos y microbiológicos de la placenta, además del feto, debido a que frecuentemente no se observan lesiones en el feto. El estudio de la placenta y de las lesiones placentarias que pueden causar abortos es una posible área de estudio en el futuro. La explicación de que muchos fetos abortados no presenten lesiones ni presencia del agente infeccioso puede ser que el aborto ocurrió como consecuencia exclusivamente de lesiones placentarias.

Esta serie de casos recolectó información útil para analizar los diferentes de patógenos, y en el futuro se deberán identificar factores de riesgo para las diferentes causas de aborto en bovinos de la cuenca lechera de Uruguay. El mejor conocimiento de las enfermedades abortivas permitirá establecer futuras estrategias de control y prevención. El conocimiento de las causas de aborto bovino en ganado de leche ayudará a futuros estudios de monitoreo y vigilancia de enfermedades reproductivas en bovinos de leche, algunas de las cuales pueden tener implicancia en la salud pública.