Caracterización genética y evolutiva del virus de la Diarrea Viral Bovina

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ÍNDICE

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RESUMEN

El virus de la Diarrea Viral Bovina (BVDV) es un patógeno de distribución mundial que causa cuantiosas pérdidas económicas al sector ganadero. La presencia de BVDV en Uruguay fue confirmada en 1996. BVDV en nuestro país es un virus endémico, con una seroprevalencia dentro de los rodeos mayor al 80%. Sin embargo, hasta el día de hoy no existe un programa para el control y la vacunación para BVDV no es obligatoria.

Al comenzar este trabajo doctoral a fines del 2015 se desconocía la incidencia de BVDV en nuestros rodeos, no se contaba con un método robusto para su detección, se desconocían los genotipos y subtipos virales circulantes y mucho menos aún su historia evolutiva. Por tanto, esta tesis planteó objetivos con la finalidad generar conocimientos en los temas mencionados sobre BVDV en Uruguay.

En el periodo 2014- 2017 se analizaron aproximadamente 3000 muestras de suero y tejidos de animales con sospecha de infección por BVD por RT-PCR y secuenciación. Con 390 muestras colectadas en 2014 y analizando la región genómica 5´UTR/Npro, se describió por primera vez la epidemiología molecular de BVDV en nuestro país. Se detectó la circulación de los subtipos BVDV-1a, BVDV-1i, y BVDV-2b en rodeos con problemas reproductivos de Uruguay, siendo el subtipo BVDV-1a el más prevalente, representando el 87.5% del total.

Posteriormente, se realizó un segundo trabajo en el que se analizaron 2600 muestras aproximadamente y las regiones genómicas 5´UTR/Npro, la proteasa Npro, y la glicoproteína de membrana E2 del virus. Se observó que el subtipo BVDV-1a continúa siendo el más prevalente en Uruguay. De acuerdo con los estudios de dinámica poblacional BVDV-1a comenzó a circular en nuestros rodeos desde 1990. Este subtipo comenzó a esparcirse y evolucionar en nuestro territorio acumulando mutaciones puntuales a una tasa de $3.48x10^{-3}$ sustituciones/sitio/año, que le permitieron adquirir características genéticas específicas que dieron lugar al surgimiento de 2 linajes genéticos BVDV-1a uruguayos. Estos linajes uruguayos, son divergentes de las cepas BVDV-1a que circulan a nivel mundial, y de la cepa vacunal NADL generalmente usada en la profilaxis contra BVDV en nuestro país. Las diferencias entre estas cepas se concentran en la glicoproteína de membrana E2, y algunas sustituciones aminoacídicas sugieren una posible falla o pérdida de neutralización por parte de los anticuerpos generados luego de la vacunación. Para tener un conocimiento más profundo, cepas BVDV-1a uruguayas fueron aisladas en cultivos celulares y sus genomas completos secuenciados. El análisis de los genomas completos de nuestras cepas confirmó la distancia genética previamente observada entre nuestras cepas BVDV-1a de campo y la cepa vacunal, lo que estaría apoyando nuestra hipótesis de una eventual necesidad de actualización de las vacunas a nivel nacional.

En suma, los resultados obtenidos en esta tesis han permitido conocer y tener información sobre BVDV en Uruguay como para elaborar un plan estratégico que permita mitigar las pérdidas

económicas causadas por este patógeno. Por otro lado, es necesario continuar investigando sobre el tema en diversos aspectos epidemiológicos, evolutivos y en estudios que aporten nuevas herramientas biotecnológicas para las vacunas y que evalúen el impacto de la vacunación.

1- INTRODUCCIÓN GENERAL

El Virus de la Diarrea viral bovina (BVDV) está diseminado a nivel mundial. BVDV es un virus económicamente importante que genera cuantiosas pérdidas económicas ya que induce desordenes reproductivos como ser: muerte embrionaria, abortos, y reducción de la fertilidad. Este virus también afecta la producción ganadera ya que causa inmunosupresión, problemas respiratorios, diarrea, y animales persistentemente infectados (PI) que son inmunotolerantes al virus (Baker, 1987; 1995; Houe, 1999).

En nuestro país hay 12 millones de cabezas de bovinos entre ganado lechero y de carne, y este número ha permanecido prácticamente incambiado desde 1998. Se cree que BVDV es uno de los factores detrás de la invariabilidad de estos números afectando la preñez y el procreo nacional (Plan Nacional en investigación en sanidad animal 2009, PLANISA). En Uruguay no hay un programa nacional para el control de BVDV, y la vacunación no es obligatoria. Un estudio realizado en el año 2000, reveló que solo el 3% de los productores vacunan contra este patógeno, y BVDV tiene una seroprevalencia de 100% en los rodeos uruguayos, y del 67% dentro de cada predio (Guarino *et al*., 2008). Estos datos fueron actualizados recientemente, obteniendo valores similares de seroprevalencia a nivel de rodeos (98.8%), pero valores más altos dentro de los establecimientos (aprox. 80%) (Dr. Federico Fernández, Ministerio de Ganadería, Agricultura y Pesca, MGAP, comunicación personal). Una extensa revisión bibliográfica realizada por Yarnall and Thrusfield en el año 2017, reveló que el impacto económico causado por BVDV oscila entre 0 a 710 dólares/animal/año, teniendo en cuenta desde las infecciones subclínicas, generalmente observadas en predios en que BVDV es endémico, y las infecciones agudas, observadas más frecuentemente en predios naïve a BVDV (Yarnall and Thrusfield, 2017).

El PLANISA elaborado en el año 2009, pone de manifiesto y plantea las necesidades a nivel Uruguay en la temática BVDV. En el año 2014 se creó la Plataforma en Salud Animal (PSA) de INIA que establece los lineamientos principales a abordar, y ayuda a llevar a cabo los cometidos y necesidades planteadas en el PLANISA. Basándonos en los lineamientos propuestos en el PLANISA y la realidad que nos encontramos en el año 2015 en la temática BVDV, como ser: carencia de métodos de detección robustos para BVDV, desconocimiento de los genotipos y subtipos de BVDV circulantes en nuestros rodeos, fue que se plantearon los objetivos de esta tesis de doctorado, buscando dar respuestas a esta temática que causa pérdidas económicas y es tan compleja para nuestra ganadería.

1.1- Biotipos virales y síndromes

Hay 2 biotipos de BVDV de acuerdo con su comportamiento en cultivos celulares: el biotipo citopático (cp) de BVDV es capaz de matar a la célula hospedera en cultivos celulares, y el biotipo no- citopático de BVDV (ncp) que en cultivos celulares no mata a la célula hospedera.

El biotipo ncp es el que se encuentra más frecuentemente infectando los rodeos, y es capaz de transmitirse entre los animales de manera horizontal y de manera vertical generando en el hospedero los síndromes que se describen a continuación:

a) Diarrea viral bovina

Es el síndrome más frecuentemente observado en la transmisión horizontal. BVDV es un virus con una alta morbilidad y generalmente una baja mortalidad, que se capta por la vía oronasal por su eliminación en la mayoría de las secreciones y excreciones como ser: orina, heces, mucus y lágrimas. Esta vía de transmisión se ve facilitada en animales que se encuentran en convivencia cercana como sucede en los tambos y en feedlots (Lértora, 2003)

El síndrome Diarrea viral bovina se caracteriza por causar una infección aguda transitoria que puede ser asintomática o presentar algunos signos clínicos como ser diarrea, inapetencia, fiebre transitoria y reducción en el rendimiento lechero. Menos frecuentemente se pueden observar infecciones agudas graves con altas tasas de mortalidad como en el denominado "síndrome hemorrágico" causado por BVDV-2 (Pellerin *et al*., 1994).

El BVDV además es capaz de inmunodeprimir a los animales porque infecta a células del sistema inmune del hospedero como son los macrófagos y linfocitos T. Además, durante la infección interfiere en la síntesis de IFN-I, obstaculizando de esta manera a las "barreras" que tiene la célula hospedera contra las infecciones, y dejando al hospedero más susceptible a otras infecciones (Baker, 1987)

Este virus también forma parte del "complejo de las diarreas neonatales" y del "complejo respiratorio bovino" junto a otros patógenos virales y bacterianos, causando enteritis y problemas respiratorios, respectivamente, afectando principalmente a animales de corta edad (Saif and Smith, 1985; Baker, 1995).

BVDV, además produce diversos trastornos reproductivos. En la Tabla 1 se muestran las principales bacterias (Brucelosis, *Leptospira*, *Trichomona*, *Neospora*, y *Campilobacter*) y virus (RIB: Herpersvirus bovino, PI3: Parainfluenza-3, y BVD (virus de la Diarrea Viral Bovina) que afectan la reproducción en bovinos provocando diversos trastornos. La afección de BVDV a la reproducción, por su impacto económico, es una de las consecuencias más "visibles" para el productor.

Enfermedad	Aborto	Reptic. servicio	Momias	Reabsor. embrion.	Celos silentes	Neonatos débiles	Mortinatos	Retenc. placent.	Metritis
Brucelos.	X	X						X	X
Leptosp.	X	X	X			X	X	X	
Trichom.	X	X		X					X
Neospor.	X	X	X		X	X	X		
Campil.	X	X		X	X				X
RIB	X	X			X				
PI3	X	X		X ?					
BVD	X	X		X		X	X		

Tabla 1. Patógenos que afectan la reproducción en bovinos. Se resaltan los trastornos reproductivos inducidos por BVDV (Tomada de http://www.prenareurogenetica.com/sindrome-reproductivo-infeccioso-bovino-srib/)

b) Animales persistentemente infectados (PI)

Es el síndrome que se produce como consecuencia de transmisión vertical del virus por su capacidad de atravesar la placenta. El feto se puede infectar ya sea porque la hembra gestante se infecta con BVDV o por el uso de un toro infectado o semen, y de esta manera infectar al feto en gestación. El virus es capaz de atravesar la placenta durante toda la gestación y las consecuencias de la infección dependerán del período de gestación en que ocurra la infección.

La infección persistente del feto se da cuando la infección ocurre entre el segundo y cuarto mes de los 9.5 meses que dura la preñez o gestación (Figura 1).

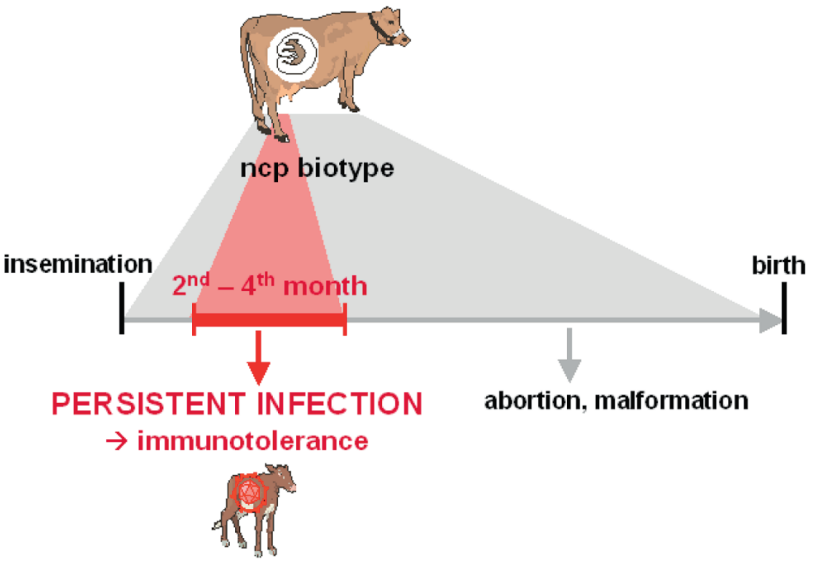

Figura 1. La infección de la hembra gestante entre el segundo y cuarto mes de preñez resulta en la parición de un ternero persistentemente infectado (PI). (Tomada y modificada de Peterhans et al., 2010)

Dada la temprana etapa de gestación en que ocurre la infección, el sistema inmune del animal en desarrollo no es capaz de montar una respuesta inmune contra el virus porque aún no se encuentra maduro, y en consecuencia el animal se hace inmunotolerante a la cepa viral con la cual está infectado (Brownlie *et al*., 1984). Estos animales PI una vez nacidos excretan permanentemente grandes cantidades del virus, diseminándolo de esta manera al resto del rodeo (Houe, 1995). Los animales PI pueden mostrar apariencia normal, signos de inmunosupresión (como ser diarrea y neumonía intermitente) y crecimiento retardado (Peterhans *et al*., 2010). Muchos animales PI mueren en su primer año de vida; sin embargo, otros alcanzan la madurez sexual y se reproducen (Baker *et al*., 1987).

c) El síndrome conocido como Enfermedad de las mucosas (EM)

Es la forma letal de la enfermedad causada por BVDV y se observa exclusivamente en los animales PI. Estos animales, luego de unos meses o años, desarrollan la EM que se caracteriza por diarrea sanguinolenta, fiebre, en algunos casos lesiones en la boca y en las membranas interdigitales, úlceras en diferentes partes del aparato digestivo, particularmente en las placas de Peyer, provocando finalmente la muerte del animal (Baker, 1995).

En animales con EM se pueden aislar los 2 biotipos de BVDV, citopático (cp) y no-citopático (ncp), que son muy similares a nivel génico y antigénico dado que el biotipo cp surge por eventos genéticos como inserción génica, duplicación, deleción o rearreglos génicos de la cepa ncp que infectó al animal de manera persistente. Las cepas cp que han sido estudiadas muestran como rasgo común una producción exacerbada de la proteína no estructural NS3. Este aumento en NS3 se asocia a una mayor síntesis de ARN viral y en cultivos celulares, a la inducción de la muerte celular por apoptosis. La proteína NS3 es considerada marcador proteico del biotipo cp (Peterhans *et al*., 2010).

1.2- Especies virales de BVDV

El BVDV pertenece a la familia F*laviviridae* del género *Pestivirus*. El Comité internacional de taxonomía viral (ICTV) reconoce 3 especies virales BVDV-1 y BVDV-2, y Pestivirus tipo HoBi o BVDV-3, recientemente renombradas como *Pestivirus* A, B, y H respectivamente (Figura 2) (ICTV, 2018).

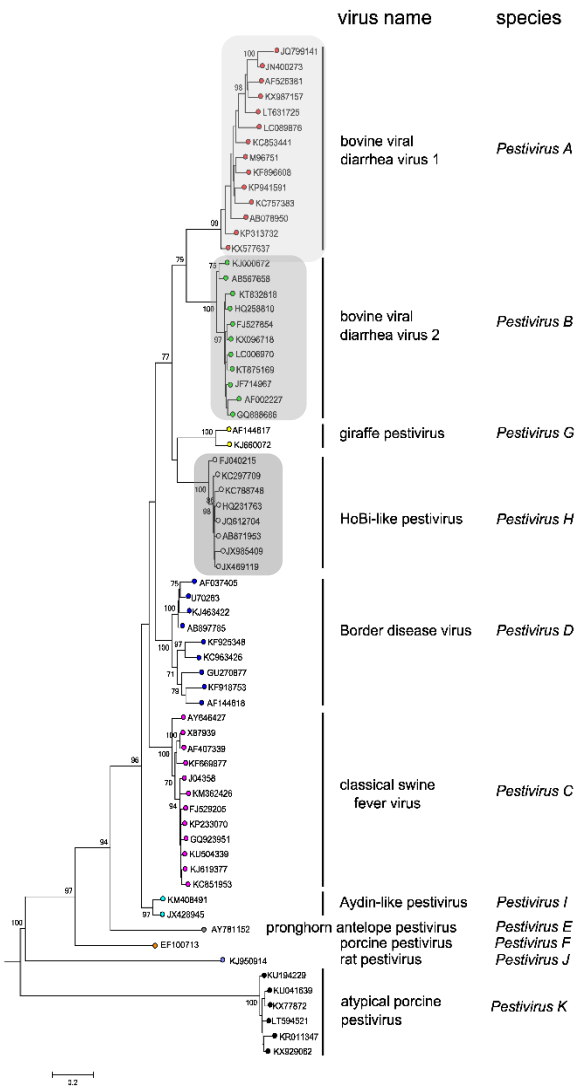

Figura 2. Árbol filogenético de las especies del género *Pestivirus* realizada con los aa 3312- 3899, usando el método de Maxima verosimilitud y el modelo de sustitución nucleotídica JTT+G con el programa MEGA6 (Tamura et al., 2013). A la derecha se encuentran los nombres de las especies. Con recuadros grises se resaltan las especies BVDV-1, BVDV-2 y *Pestivirus* tipo HoBi. (Tomado y modificado del informe de ICTV de año 2018)

1.3- Organización del genoma de BVDV y proteínas virales

El genoma de BVDV es ARN simple hebra de polaridad positiva de 12.3 kb de largo aproximadamente. El genoma de BVDV tiene un único marco abierto de lectura (ORF) que codifica para una poliproteína que luego es clivada en las proteínas individuales. En ambos extremos el genoma tiene regiones no traducidas (UTRs), 5´UTR y 3´UTR, respectivamente (Figura 3) (Lindenbach *et al*., 2007).

Figura 3. Esquema que muestra la organización del genoma de BVDV y las proteínas para las cuales codifica la poliproteína viral. (Tomada de http://viralzone.expasy.org)

Ambas UTR se pliegan formando estructuras secundarias que interactúan con proteínas virales y celulares durante la replicación, transcripción y traducción del genoma viral, ya que el genoma del virus carece de las estructuras características de los ARN mensajeros celulares como ser el cap en el extremo 5´y la cola poliA en el extremo 3´ (Pestova *et al*., 1999; Yu *et al*., 2000; Isken *et al*., 2004; Moes *et al*., 2007). La 5´UTR se pliega en una estructura secundaria denominada IRES (sitio de entrada interno para ribosomas) que es reconocida por proteínas celulares que participan en la traducción y dirigen al ribosoma hacia el codón de inicio AUG correcto en el marco abierto de lectura (ORF).

1.4- Proteínas virales y estructura de la partícula viral

BVDV tiene un único ORF que codifica para una poliproteína que es procesada post traduccionalmente por las proteasas virales y celulares dando lugar a 11-12 proteínas estructurales y no- estructurales (Figura 4).

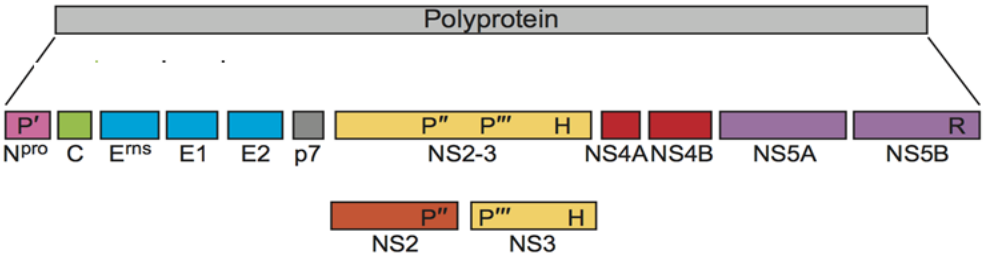

Figura 4. Organización genómica de BVDV, y procesamiento de la poliproteína en proteínas estructurales y no-estructurales (Tomada y modificada de Informe de ICTV)

El virus codifica 4 proteínas estructurales: la proteína del Core (C) que forma la nucleocápside y 3 glicoproteínas de membrana: Erns, E1 y E2; y 8 proteínas no- estructurales: la autoproteasa viral (N^{pro}), la proteasa viral (NS2-3), el co-factor de la proteasa viral (NS4A/B), la proteína que forma parte del complejo de replicación del genoma viral (NS5A), la ARN polimerasa ARN dependiente (NS5B) (Figura 3) (Lindenbach *et al*., 2007).

La partícula viral de BVDV se compone de una región de core formada por el ARN genómico rodeado de la proteína de cápside C. Este core está rodeado por una bicapa lipídica que forma la envoltura

viral que tiene las glicoproteínas virales insertas en ella (Figura 5). El virión mide entre 40 -60 nm de diámetro (Neill *et al*., 2013)

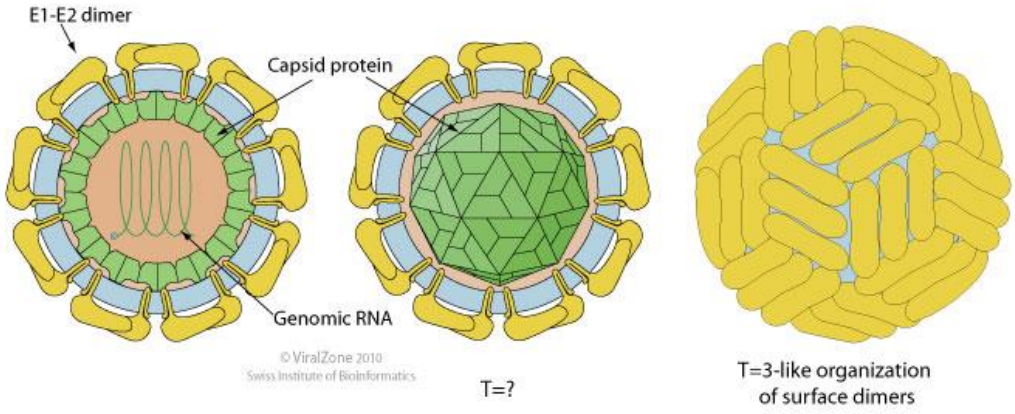

Figura 5. Estructura de la partícula viral de BVDV. (Tomada de http://viralzone.expasy.org/)

1.5- Variabilidad genética de BVDV

BVDV durante su ciclo replicativo posee distintos mecanismos moleculares que le confieren una importante capacidad de variabilidad genética. Los principales mecanismos son: a) la mutación puntual, b) la recombinación homóloga y c) recombinación no- homóloga.

a) Mutación puntual: especies y subtipos virales de BVDV

El ICTV reconoce 3 especies virales: BVDV-1 (*Pestivirus A*), BVDV-2 (*Pestivirus B*) y el Pestivirus tipo HoBi (*Pestivirus H*) (ICTV, 2018). Si bien el ICTV no contempla la división de las especies, la realidad es que éstas se han ido diversificando en subtipos virales. La emergencia de los diferentes subtipos virales dentro de cada una de las especies de BVDV se debe a la acumulación de mutaciones puntuales por la incapacidad de su ARN polimerasa ARN dependiente de corregir errores. Distintas regiones del genoma de BVDV evolucionan a diferentes tasas de sustitución nucleotídica/sitio/año, siendo la 5´UTR la región más conservada con tasas de sustitución nucleotidica/sitio/ año calculadas en el orden de 10^{-4} (Luzzago *et al*., 2012; Chernick *et al*., 2014). La glicoproteína de membrana E2 se une al receptor celular CD46 en macrófagos y linfocitos (Maurer *et al*., 2004). La E2 tiene los mayores determinantes antigénicos que se ubican en el extremo amino terminal de la proteína, y por tanto la mayor parte de la respuesta humoral del hospedero está dirigida a esta glicoproteína de membrana (Deregt *et al*., 1998). Debido a esta presión selectiva ejercida por el sistema inmune del hospedero las glicoproteínas de membrana E1-E2 tienen una tasa de sustitución nucleotídica/sitio/año de un orden mayor a la de la 5' UTR, del orden de 10^{-3} (Chernick *et al*., 2014).

La diversidad genética de BVDV ha sido estudiada mediante análisis filogenéticos a partir de secuencias nucleotídicas de diferentes regiones del genoma viral, como ser la 5´UTR, la glicoproteína

de membrana E2, la autoproteasa N^{pro}, y la proteasa NS3 (Vilceck *et al*., 1994; Ridpath *et al*., 1994; Becher *et al*., 1997; Weber *et al*., 2015).

De esta manera se ha determinado que la especie BVDV-1 es la más diversa genéticamente y al momento se han descrito 23 subtipos virales denominadas BVDV-1a al 1w (Giammarioli *et al*., 2015; Jackova *et al*., 2008; Luzzago *et al*., 2014; Nagai *et al*., 2008; Vilcek *et al*., 2001; Xue *et al*., 2010 y Deng *et al*., 2015; 2020). La diversidad de subtipos virales de esta especie viral ha sido descrita en Europa y Asia (Yesilbag *et al*., 2017).

El BVDV-2 y el *Pestivirus* tipo HoBi serían menos divergentes y al momento se han reportado 4 subtipos virales para cada una de estas especies virales denominadas como BVDV-2a al 2d (Vilcek *et al*., 1994; Flores *et al*., 2002; Peterhans *et al*., 2010; Jenckel *et al*., 2014); y para el Pestivirus tipo HoBi "a" a "d" (Mishra *et al*., 2014; Giammarioli *et al*., 2015; Silveira *et al*., 2020). La especie BVDV-2 y sus subtipos parece estar más presente en las américas (Yesilbag *et al*., 2017). El Pestivirus tipo HoBi aparentemente tendría una distribución más limitada que las otras 2 especies, y sus subtipos virales ya que ha sido descrita su presencia solo en determinados países como ser India (Mishra et al., 2014), Italia (Decaro *et al*., 2012; Giammarioli et al., 2015), Brasil (Weber et al., 2016; Cruz et al., 2018; Silveira S. *et al*., 2020) y Argentina (Pecora *et al*., 2019).

b) Recombinación

La recombinación homóloga y no- homóloga también son mecanismos moleculares generadores de variabilidad genética en el genoma de BVDV.

La recombinación no homóloga del ARN es la responsable de la emergencia del biotipo cp en los animales PI a partir de una cepa ncp. De esta manera es que se han descrito diversos tipos de secuencias de ARN viral o fragmentos de ARN del hospedero en el genoma de las cepas del biotipo cp (Peterhans *et al.*, 2010). Este tipo de recombinación se ha observado de manera asociada a la replicación por el cambio de molde por parte de la ARN polimerasa dependiente de ARN, o bien de manera independiente a la replicación (Gallei *et al*., 2004). Estos eventos de recombinación que dan lugar a inserciones en el genoma de las cepas cp son únicas de cada cepa cp que emerge. Las cepas cp no se transmiten entre animales, por lo que cuando emergen son conocidas como "virus emergentes condenadas a su extinción" (Peterhans *et al*., 2010).

También se han descripto en BVDV, aunque son poco frecuente, eventos de recombinación homóloga entre cepas del mismo o de diferente subtipo viral (Jones *et al*., 2004; Weber *et al*., 2015; Kővágó *et al*., 2016). No se han descrito cepas recombinantes infectantes, por lo que son eventos aislados que no participan en la emergencia de las nuevas especies o genotipos virales de BVDV (Bolin *et al*., 2004).

1.6- Medidas de control para BVDV

Los países que han implementado planes de saneamiento para BVDV han usado estrategias que se basan en: vigilancia continua del virus, eliminación de los animales PI y prevención de nacimiento de animales con esta condición, controles antes de movilizar animales para no introducir animales infectados en predios libres de BVDV, y vacunación (Moennig and Becher, 2018).

a) Detección de BVDV y vigilancia

Muchos métodos han sido desarrollados para la detección de BVDV, incluyendo: aislamiento viral (VI), inmunohistoquímica (IHC), retrotranscripción - amplificación en cadena de la polimerasa (RT-PCR), y ensayo inmunoabsorbente ligado a enzima de captura de antígeno (ACE). Cada una de estas pruebas tiene sus fortalezas y debilidades en cuanto a sensibilidad, costos, cuan laboriosas son, y el tiempo que insumen. De los 4 métodos, para la vigilancia de BVDV en los rodeos, el método más apropiado es la PCR en tiempo real ya que es una técnica rápida y sensible, que permite testear la presencia del antígeno a partir de diversas matrices como ser: suero, sangre, tanque de leche, semen, y tejidos. Además, las muestras de suero y/o sangre de un mismo rodeo pueden ser agrupadas para su análisis, reduciendo así los costos de manera significativa (OIE, Manual terrestre 2018).

La detección de BVDV sirve para detectar animales PI. Estos animales PI, como ya se mencionó anteriormente, son excretores continuos del virus diseminándolo al resto de los animales del rodeo, por lo que deben ser eliminados de los rodeos.

La detección molecular de BVDV, también sirve para realizar vigilancia continua de BVDV y controlar a los animales antes de movilizarlos para no introducir animales infectados en predios libres del virus.

b) Vacunación

La vacunación es la medida de control preferida para el control de una determinada enfermedad debido a su efectividad y bajo costo. De preferencia se utilizan las vacunas vivas modificadas (MLV) dado que rememoran la infección natural y por tanto se logra una respuesta inmune del tipo humoral y celular dirigida a E2 y NS23 principalmente, y en menor medida contra E1 y Erns (Bolin and Ridpath, 1989). El problema al que nos enfrentamos al trabajar con BVDV, es que las vacunas MLV no son recomendadas para el uso de hembras preñadas dado que el virus vacunal, al igual que el virus de campo, es capaz de atravesar la placenta, y se observaron algunos casos de Enfermedad de las mucosas e inmunodepresión post vacunación (Ridpath, 2013). Las nuevas vacunas MLV se hacen usando cepas citopáticas de BVDV, ya que este biotipo no es capaz de producir animales PI (Ridpath, 2013).

Las vacunas con virus inactivado, también denominadas vacunas a virus muerto (KVs), pueden ser usadas a cualquier edad y en cualquier etapa de la gestación. Estas vacunas deben usar adyuvante

para la presentación del antígeno, y desarrollan inmunidad del tipo humoral principalmente dirigida a E2 y la inmunidad celular que generan es variable, que puede ser desde incompleta a fuerte (Ridpath, 2013; Moennig and Becher, 2018).

1.7- Antecedentes en Uruguay al 2015 y justificación de tesis de Doctorado

Se presume que BVDV está presente en Uruguay desde 1980. Sin embargo, su presencia se confirmó mediante inmunohistoquímica en 1996 (Saizar *et al*., 1998; Guarino *et al*., 2008). En un estudio representativo de todos los rodeos del país realizado con muestras de suero colectadas durante el año 2000, se determinó que BVDV está presente en el 100% de los rodeos y tiene una seroprevalencia de 67,4% dentro de los mismos (Guarino *et al*., 2008). Actualmente la seroprevalencia en ganado de carne es de 82,8% en ganado de carne y 80,9% en ganado de leche (Dr. Federico Fernández, Ministerio de Ganadería, Agricultura y Pesca, MGAP, comunicación personal)

En Uruguay, la problemática o incidencia de BVDV ha sido, por lo general, subestimada ya sea porque generalmente se presenta la infección aguda transitoria con signos clínicos leves, o porque los problemas reproductivos en los que BVDV tiene gran impacto son atribuidos, generalmente, a causas nutricionales, según la información recabada en el documento "Propuesta de líneas de investigación y acciones para el Plan Nacional de Investigación en Salud Animal" (PLANISA) que fue presentado en setiembre de 2009. El PLANISA fue el primer Plan Nacional de Investigación en Salud Animal, elaborado de y para uruguayos, con la conjunción de instituciones y la participación de técnicos, investigadores, productores, industriales, actores públicos y privados con el cometido de establecer las principales líneas de investigación en salud animal para el período 2009-2013. De acuerdo con el PLANISA las enfermedades reproductivas se encuentran dentro de las 5 enfermedades principales que afectan al ganado de carne y leche. El PLANISA, apoyándose en la alta seroprevalencia de BVDV en nuestro país destaca que la Diarrea viral bovina debe ser una de las enfermedades a estudiar.

El PLANISA dice de BVDV, y de otras enfermedades reproductivas:

- Tienen deficiencias en sus métodos de diagnóstico
- No se cuentan con vacunas eficaces
- No están claras las mejores estrategias de prevención
- Por tanto, la investigación en ellas debería aportar numerosas respuestas

El PLANISA en el año 2013 pasó a llamarse CCISA (Comité de coordinación en investigación en Salud Animal). Este comité apoyó al grupo de asesoramiento estratégico contratado por la Dirección Nacional de INIA para el diseño de su Plataforma de Salud Animal (PSA) que se creó en el año 2014. La PSA tuvo el cometido inicial de priorizar 3 líneas de investigación:

- Resistencia a parasitaria

- Mortalidad de terneros
- Enfermedades infecciosas de la reproducción en bovinos

De estas 3 líneas de investigación, BVDV tiene impacto en las últimas 2. La PSA y la ANII crearon el Fondo Sectorial en Salud Animal, los proyectos de investigación que se denominaron fondos de investigación INNOVAGRO. De esta manera la PSA de INIA financió proyectos de investigación relacionados a las 2 primeras líneas de investigación; y la PSA y ANII financiaron proyectos de investigación vinculados a la tercera línea de investigación.

Nuestro grupo ganó un proyecto Innovagro intitulado "Estudio de la epidemiología molecular y prevalencia serológica del virus de la Diarrea Viral Bovina en rodeos de carne y leche en el Uruguay con el fin de seleccionar cepas para una vacuna protectora de alcance masivo". Ese proyecto compartió objetivos con esta tesis de doctorado y además, permitió financiar y hacer posible el trabajo de investigación realizado.

Basados en los antecedentes en la temática BVDV descritos anteriormente y la necesidad de estudiar en profundidad tanto el grado de afectación a nivel país como las características epidemiológicas y evolutivas de BVDV en Uruguay, fue que se establecieron los objetivos de esta tesis.

2- HIPÓTESIS DE TRABAJO

1- Uruguay mantiene una estrecha relación comercial con sus países vecinos Argentina y Brasil, y por tanto es de esperar:

- Que en Uruguay la situación epidemiológica sea similar a la de Argentina y Brasil, y por tanto que circulen las 3 especies virales de BVDV, BVDV-1, BVDV-2, y Pestivirus tipo HoBi
- Que las cepas uruguayas de BVDV sean similares genéticamente a las de Argentina y Brasil

2- En la formulación de las vacunas Uruguayas se encuentra la cepa NADL del subtipo BVDV-1a que data de la década del 80´, por tanto es de esperar que las cepas uruguayas de BVDV de ese subtipo viral sean divergentes respecto a la cepa vacunal.

3- OBJETIVOS

2.1- Objetivo general

Caracterización genética y evolutiva del virus de la Diarrea Viral Bovina (BVDV) en Uruguay

2.2- Objetivos específicos

- ***Objetivo específico 1*:** Desarrollo de un método robusto para la detección molecular de BVDV-1, BVDV-2 y BVDV-3.
- ***Objetivo específico 2*:** Estudiar el grado de variabilidad genética y la diversidad viral existente en las cepas Uruguayas, respecto a las cepas de la región y el resto del mundo.
- ***Objetivo específico 3*:** Realizar estudios de coalescencia para conocer y reconstruir la historia evolutiva de BVDV en la región y el mundo.
- ***Objetivo específico 4*:** Realizar análisis comparativos de las cepas de campo uruguayas de BVDV con las cepas vacunales utilizadas en la profilaxis contra BVDV en Uruguay
- ***Objetivo específico 5*:** Realizar el aislamiento de cepas de campo potencialmente promisorias a ser utilizadas en una vacuna.

4- CAPÍTULOS

Capítulo 1_ Manuscrito 1:

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 1613:529–535.
DOI: 10.1007/s00705-015-2688-4

En este artículo se analiza la seroprevalencia y prevalencia de BVDV en 390 animales no vacunados provenientes de 14 predios con fallas reproductivas.

También se describe por primera vez en Uruguay la diversidad genética de las cepas de BVDV.

Los principales hallazgos de este trabajo fueron los siguientes:

- Todos los predios estudiados (n=14) fueron seropositivos a BVDV, y una seroprevalencia de 76.4%, 298 animales seropositivos de un total de 390.
- El 4.1% (n= 16) de los animales fueron positivos a BVDV por PCR en Tiempo real.
- La genotipificación de las cepas positivas reveló que en nuestros rodeos están presentes la especie BVDV-1, subtipos BVDV-1a y BVDV-1i; y la especie BVDV-2, subtipo BVDV-2b.
- El subtipo mayoritario encontrado en nuestro muestreo fue BVDV-1a
- Las cepas Uruguayas BVDV-1a (n=12) fueron similares a algunas cepas de este subtipo viral originarias de Brasil.
- La cepa del subtipo BVDV-1i (n=1) encontrada en Uruguay fue un evento inesperado ya que se creía que la circulación de ese subtipo estaba limitada a Reino Unido.
- La cepa BVDV-2b (n=1) se agrupó con las cepas de referencia de este subtipo viral, no guardando similitud con cepas de campo regionales.

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ORIGINAL ARTICLE

Molecular diversity of bovine viral diarrhea virus in uruguay

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Abstract Bovine viral diarrhea (BVD) affects bovine production and reproduction causing significant economic losses all over the world. Two viral species has been recognized: BVDV-1 and BVDV-2, both distributed worldwide. Recently, novel specie of BVDV named HoBi-like pestivirus was discovered. The presence of BVDV was confirmed in 1996 in Uruguay, however, does not exist until today a schedule of compulsory vaccination along the country. Serological studies with samples from all Uruguayan herds were performed during 2000 and 2001 demonstrating that all of them were seropositive to BVDV with a mean prevalence of 69 %. In addition, there have been no new studies done since those previously described and it is important to mention that the genetic diversity of BVD has never been described in Uruguay. Nowadays, there is strongly suspect that BVDV is one of the most important causes of reproductive failures in our herds. The aim of this study was to describe for the first time in Uruguay the genetic diversity of BVDV with samples collected from different regions along the country. Serological status of 390 non-vaccinated animals against BVDV with reproductive problems from farms of Rivera, Tacuarembó and Florida departments of Uruguay were studied. All herds were seropositive to BVDV and high proportion of animals were positive (298/390), while 4.1 % (16/390) of the animals were positive to Antigen Capture ELISA test and Real Time PCR. Phylogenetic analysis performed with concatenated sequences from the 5′UTR and Npro genomic regions revealed that BVDV-1 and BVDV-2 are infecting our herds, being BVDV-1 the most frequently found. The major subtype was BVDV-1a, followed by BVDV-1i and BVDV-2b. This is the first study that describes the genetic diversity of BVDV in Uruguay and it will contribute to the elaboration of sanitization programs.

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Abbreviations

ACE	Antigen capture ELISA
Bp	Base pair
BVD	Bovine viral diarrhea
BVDV	Bovine viral diarrhea virus
ELISA	Enzyme-linked immune sorbent assay
ORF	Open reading frame
PCR	Polymerase chain reaction
UTR	Untranslated region

Introduction

Bovine viral diarrhea (BVD) is an economically important disease causing a wide range of clinical signs and reproductive failures [12]. Bovine Viral Diarrhea Virus (BVDV) is transmitted horizontally by secretions and excretions

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(such as tears, mucus, feces) and animal management (gloves, syringes) inducing reproductive disorders (abortion, delayed calving and reduce fertility) and affecting productivity by its immune depressive effects, respiratory problems and diarrhea [1, 2]. BVDV it is also transmitted vertically due to its ability to cross the placenta in pregnant dams causing abortion, malformations and inmunotolerant persistent infected (PI) calves [12]. These PI animals are the major disseminator of the virus in herds spreading it to other susceptible animals [11]. In infected herds, the prevalence indices of PI animals vary between 0.5 to 2 % [12]. PI animals usually die during the first two years of life from the fatal form of the disease "mucosal disease", or due to other diseases mostly as a consequence of immune depression cause by the virus [2, 18].

BVDV belongs to the *Pestivirus* genus into the *Flaviviridae* family, and their genome is a single stranded positive sense RNA of 12.3 Kb approximately in length flanked at both ends by untranslated regions (UTR). The only open reading frame (ORF) of BVDV codifies for a single polyprotein that is cleaved post translationally into structural and non-structural proteins [19].

Different genomics regions of BVDV have been used to study the genetic diversity and evolution of the virus, including the 5′UTR, the glycoprotein E2, auto protease Npro and NS3 protease [4, 24, 29, 30]. There are 2 species recognized by the International Committee of Taxonomy Virus (ICTV): BVDV-1 and BVDV-2, both distributed worldwide [13]. BVDV-1 genotype is the most genetically diverse, being described twenty subtypes, BVDV-1a to BVDV-1t [8, 14, 17, 20, 32, 35]. Nevertheless, only two subtypes of BVDV-2 (BVDV-2a and BVDV-2b) have been described [7]. Recently, HoBi-like pestivirus has been proposed as a new species of the genera based on its genetic and antigenic closeness to BVDV-1 and BVDV-2 [3, 16, 26].

Uruguay is located in South America bordered by Argentina and Brazil. According to official data, cattle population in Uruguay is approximately 11.5 million and beef and milk production are among the main products for the Uruguayan economy. The total numbers of bovine population have remained almost unchanged since 1998, and the calving rate, in the past seventeen years had reached values as low as 59.2 % and the upper rate reached was 71.4 % [6]. Producers and technician related to area have shown concern about this numbers and it is suspected that BVDV could be one of the major contributors to this situation [23].

In Uruguay, BVDV seems to be circulating since the eighties, although its presence was not confirmed until 1996 by immunohistochemistry techniques [25]. There is no prophylaxis schedule for BVDV and a research done about fifteen years ago revealed that only 3 % of the producers implement some vaccine against it [9].

Serological studies of Uruguayan herds during 2000 and 2001 revealed that all herds had BVDV seropositive cattle with a mean prevalence of 69 % [9]; nevertheless, the genetic diversity of BVDV has never been described in Uruguay.

The aim of this study was to describe for the first time in Uruguay the genetic diversity of BVDV with samples collected from different regions along the country from herds having reproductive problems.

Materials and methods

Sample collection

During 2014, 390 animals ranging 6 month to one year old that belong to 14 farms from the departments of Tacuarembó (n = 5), Rivera (n = 2), (Beef farms) and Florida (n = 7) (dairy farming) of Uruguay, were collected. Animals came from herds with reproductive problems and where no vaccination schedule against the disease was applied. The herds belong to northern and southern geographic regions of Uruguay. The animals showed a wide range of clinical manifestations characteristic of BVD such as weakness, fever, diarrhea, affected growth rate and nervous problems like muscle shaking and incoordination. Blood serum samples were collected and then remitted to the Faculty of Veterinary, University of the Republic and store at −20 °C until be tested by indirect and direct ELISA.

Sample screening by indirect and capture ELISA

In order to detect BVDV antibodies in serum samples an Antibody enzyme-linked immunosorbent assay (IDEXX BVDV total Ab – Switzerland) was done following the manufacturer's protocol.

Antigen screening of serum samples was done by an Ems antigen capture ELISA (ACE) (IDEXX- Switzerland) performed following manufacturefs instructions.

Real Time PCR

Viral RNA was extracted from blood serum samples using QIAamp viral RNA mini Kit (QIAGEN®, Germany) following instructions specified by the manufacturer. Reverse transcription was made using random primers and SuperScript II enzyme (Invitrogen™, USA). Real time PCR assays targeting 207 bp of 5′UTR region of BVDV were performed using primers BVDV190F and V326, and TaqMan® probe TQ-Pesti described by Hoffman et al. (2006)

and Gaede et al. (2005), respectively. In order to detect BVDV-1, BVDV-2 and HoBi-like pestivirus, some bases of primers and probe were degenerated for this work as it is shown in bold type letter in Table 1. All Real Time PCR reactions were performed with SensiMix™ II Probe Kit (Bioline Reagents Ltd.) and Rotor-Gene Q instrument (Qiagen®) following manufacture's recommendation. The following temperature profile was used: 10 min at 95 C (activation *Taq* polymerase), followed by 45 cycles of 10 s at 95 C (denaturation), 60 s at 50 C (annealing and extension).

Amplification by conventional PCR

With the aim to genotype positive samples of BVDV previously detected by Real Time PCR, 288 bp of the 5′UTR of the virus were amplified using primers V324 and V326 designed by Vilcek et al. (1994) that were degenerated in this study as it is shown in Table 1. PCR reactions were performed using 1U of Taq DNA Polymerase (Invitrogen®) following thermal cycling PCR conditions described by Vilcek et al. (1994). A second round of PCR using 5′UTR Real time PCR primers and the same PCR conditions described above was done to positive samples in which it was not possible to get an amplicon.

A hemi nested PCR was performed to get an amplicon of 424 bp of the Npro gene as follows: first round of PCR amplified a fragment of 738 bp from Npro and a part of the C coding region using primers BD1 and BD2 described by Vilcek et al. (1997). The second round of PCR, hemi nested PCR, which amplified a fragment of 424 bp was done using primer BD1 and BD3, the former one described by Vilcek et al. (2001). All PCR reactions were performed using 1U of Taq DNA Polymerase (Invitrogen®). First round of PCR was performed with 35 cycles: 1 min at 95 °C (denaturation); 45 s at 52 °C (annealing); and 30 s at 72 °C (extension). The second round of PCR was performed at the same thermal conditions over 30 cycles.

Positive amplicons to 5′UTR and Npro were purified by QIAquick PCR purification kit (QIAGEN®, Germany) and both strands were sequenced on the automated sequencer ABI3130 Genetic Analyzer (Applied Biosystems®, Foster City, CA, U.S.A.) by the DNA Sequencing Service of Institute Pasteur of Montevideo. Sequences were edited and assembled using DNASTAR software. The origin and the genotyping of the Uruguayan strains are summarized in Table 2.

Phylogenetic analysis

In order to achieve better bootstrapped nodes in the phylogenetic analysis, 5′UTR and Npro sequences were concatenated into a fragment of 607pb length and were aligned along with BVDV representative strains of BVDV-1, BVDV-2 and HoBi-like pestivirus genotypes and subtypes retrieved from GenBank database. Accession numbers of reference sequences were putted as 5′UTR/Npro following the name of the strain in the phylogenetic reconstruction. Reference sequence of Border disease virus (BDV) was used as out-group. The evolutionary model that best fitted the data set was chosen based on jmodelTest software results. Phylogenetic tree was constructed by Maximum likelihood (ML) method and in order to test its statistical significance Bootstrap method was carried out (1000 replicates) using Mega version 6 [28].

Results

Of the total number of animals tested, 76.4 % (298/390) were seropositive to BVDV. All 14 herds resulted to be seropositive. Eleven samples were positive to BVDV antigen by ACE and negative to indirect ELISA test at the time of sampling. These positive samples were confirmed by Real Time PCR. On the other hand, 14 additional samples that were nearly the cutoff value of ACE and were negative to indirect ELISA test, were assessed by Real time PCR and five of them resulted to be positive to BVDV. In summary, a total of 16 (4.1 %) positive samples out of 390 serologically tested samples were detected by real time

Table 1 Primers and probe used in PCR experiments in this study

Name of primer/probe and its sequence	Reference
BVDV190Fmod 5′ FAM- GDAGTCGTCARTGGTTCGAC-3′	Modified of Hoffman et al. 2006
V324mod 5′- ATGCCCWTAGTAGGACTAGCA-3′	Modified of Vilcek et al. 1994
V326mod 5′-WCAACTCCATGTGCCATGTAC-3′	Modified of Vilcek et al. 1994
TQ-Pesti probe mod 5′-TGCYAYGTGGACGAGGGCRTGC-3′	Modified of Gaede et al. 2005
BD1: 5′- TCTCTGCTGTACATGGCACATG-3′	Vilcek et al. 1997
BD2: 5′- TTGTTRTGGTACARRCCGTC-3′	Vilcek et al. 1997
BD3: 5′- CCATCTATRCACACATAAATGTGGT-3′	Vilcek et al. 2001

Modified bases in this work are shown in bold type, where D is G, A and T; R is G or A; W is T or A; and Y is T or C

Table 2 BVDV positive strains from this study, its origin, accession number and BVDV typing

Strain	Region of isolation	Acc number 5′UTR&Npro	BVDV type
408TboUY/072014	Tacuarembó	KT833787	BVDV-1a
409TboUY/072014	Tacuarembó	KT833788	BVDV-1a
429TboUY/082014	Tacuarembó	KT833789	BVDV-1a
430TboUY/082014	Tacuarembó	KT833790	BVDV-1a
431TboUY/082014	Tacuarembó	KT833791	BVDV-1a
432TboUY/082014	Tacuarembó	KT833792	BVDV-1a
433FaUY/032014	Florida	KT833793	BVDV-1a
434FaUY/032014	Florida	KT833784	BVDV-1a
435FaUY/032014	Florida	KT833794	BVDV-1a
436FaUY/052014	Florida	KT833795	BVDV-1i
437TboUY/042014	Tacuarembó	KT833796	BVDV-1a
438TboUY/042014	Tacuarembó	KT833797	BVDV-1a
439RvUY/082014	Rivera	KT833799	BVDV-2b
653TboUY/082014	Tacuarembó	KT833798	BVDV-1a
651TboUY/082014	Tacuarembó		Not genotyped
652UYTbo/082014	Tacuarembó		Not genotyped

PCR and belonged to Tacuarembó (n = 11), Florida (n = 4) and Rivera (n = 1).

Amplicons obtained from the 5′UTR and Npro genomic regions from 14 of the 16 detected samples, were successfully sequenced and then edited, concatenated and analyzed by phylogenetic methods. Strain number, geographic region, accession number and genotype, are listed in Table 2. The best fitted nucleotide evolutionary model for the dataset obtained in this study was GTR plus gamma according to jModeltest. The phylogenetic reconstruction showed that Uruguayan strains were separated in 2 clusters. As it can be seen in Fig. 1, 13 out of 14 local strains grouped into BVDV-1 genotype and only one strain 439RvUY/081014 clustered into BVDV-2, being both clusters well supported with high bootstrap values of 93 % and 100 %, respectively. Most BVDV-1 Uruguayan strains (n = 12) clustered together in BVDV-1a subtype (98 % of bootstrap value). This BVDV-1a group was subdivided in 2 subgroups whose genetic similarity was 99.1 %. The mayor subgroup had 99.7 % of nucleotide similarity among its 9 sequences (408, 409 TboUY/072014; 429, 430, 432TboUY/082014; 433-435FaUY/032014 and 653TboUY/082014). This subgroup included sequences typed as BVDV-1a from Brazil (LV17-09/13 and LV60-53/13). The other subgroup of BVDV-1a was less heterogeneous and sequence diversity between 431TboUY/082014, 437 and 438TboUY/042014 were 0.05 %. 436FaUY/052014 belonged to subtype BVDV-1i as clustered along with strain 23-15 of this subtype bootstrapped by 83 %. Only one local strain, 439RvUY/082014 grouped in BVDV-2 genotype, and was particularly related to BVDV-2b reference strains named Soldan and Giessen 6.

Discussion

Surveillance of BVDV by serological techniques in non-vaccinated herds is a very useful and powerful tool because it gives valuable information about individual animal health and herd status. Positive results mean that there are animals that have been previously exposed to the virus and it is also indicative that those positive animals are not persistent infected. At a herd level, to have a high proportion of seropositive animals it is also indicative that the animals are in direct exposure to the virus and probably due to the presence of PI animals in the herd disseminating the virus. PI animals of BVDV can be diagnosed based on negative results to Indirect ELISA and positive results to antigen BVDV presence by direct ELISA or by molecular techniques such as Real Time PCR.

In the present study 390 animals belonging to 14 different herds with reproductive problems were screened by indirect ELISA and 76.4 % of them were seropositive. At herd level, 100 % of the herds tested in this study were serologically positive to BVDV. Previous studies made in our country in period 2000-2001, representative of all Uruguayan herds, denoted that 100 % of herds were sero-positive [9].

Of all animals tested during the present work, 4,1 % were positive to BVDV by real time PCR. Since all of these animals were negative to indirect ELISA test at the time of sampling, they could be either having an acute transient infection with BVDV or they could be PI animals since its youthfulness and high percentage of seropositive animals. This percentage of animals with acute infections is in agreement with previous studies done in Belgium in herds with reproductive problems [10].

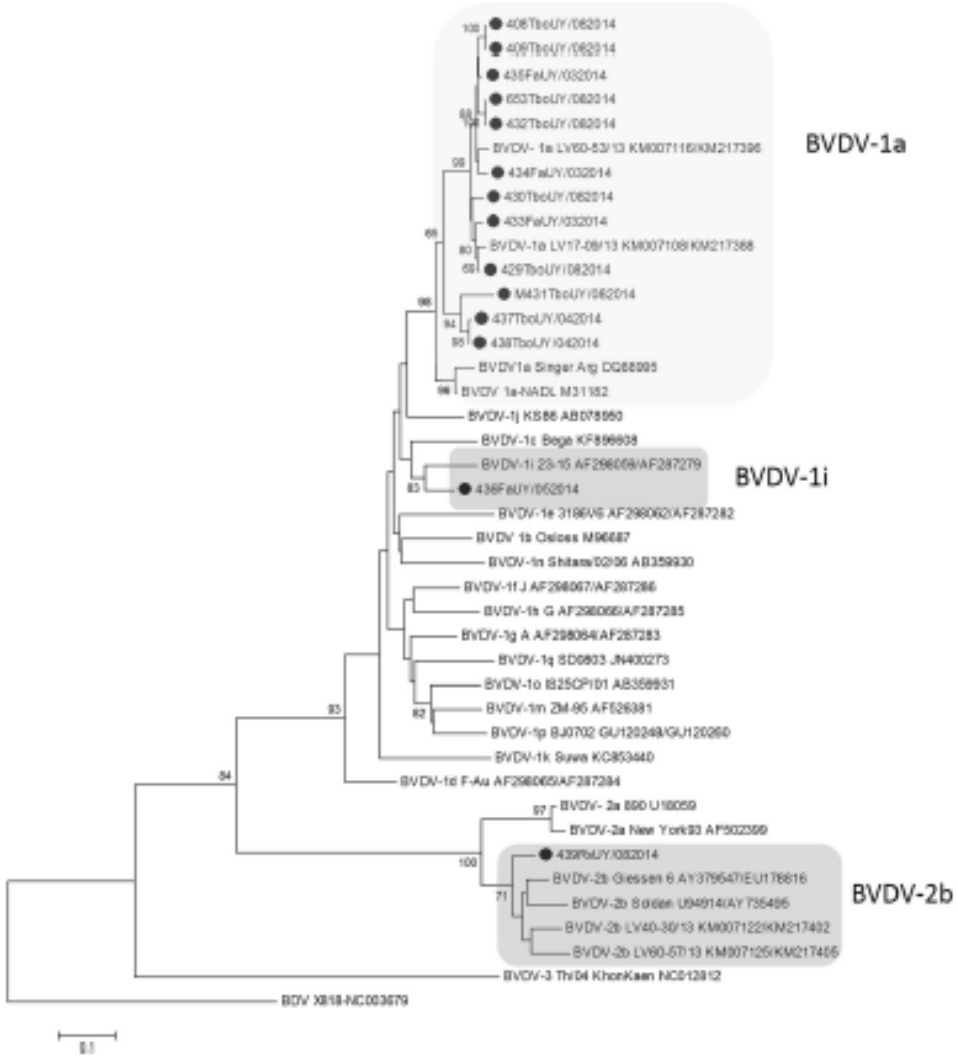

Fig. 1 Phylogenetic tree constructed by the Maximum likelihood method using 607 nt of the concatenated sequences of 5′UTR and Npro of BVDV genome. Bootstrap (1000 replicas) are indicated at internal nodes. Uruguayan strains are highlighted by dots. Reference sequences of genotypes BVDV-1, BVDV-2 and HoBi-like pestivirus were retrieved from Genbank for comparison purposes. BVD (Border disease virus) sequence was included in the analysis as an out-group

Molecular genotyping studies were done in Argentina, Brazil and Chile, most of them with partial sequence of 5′UTR and the viral glycoprotein E2, detecting the 3 genotypes of BVDV: BVDV-1, BVDV-2 and HoBi-like virus in these countries [5, 7, 15, 22, 33]. Weber et al. (2014) were the only one that analyzed 5′UTR and Npro genomic regions, thus being the only regional isolates included in the present phylogenetic analysis.

Genotyping analysis of the 14 positive BVDV Uruguayan samples, revealed that BVDV-1 and BVDV-2 circulates in our herds, being BVDV-1 the most prevalent genotype with a frequency of 92.9 % while one strain, 439RvUY/082014, were genotyped as BVDV-2 representing 7.1 % of the tested samples. BVDV-1 genotype it is also highly frequently in Argentina, while in Brazil and Chile the situation is different as both genotypes are distributed almost at equal frequencies [21, 22, 34]. In Uruguay as well as in Brazil [34], among BVDV-1 genotype, the most frequent subtype was BVDV-1a 92.3 % (n = 12). The major subgroup of BVDV-1a Uruguayan strains were highly similar to Brazilian strains LV17-09/13 and LV60-53/13 since it clustered together with 99 % of bootstrap value. In Argentina BVDV-1a was the predominant subtype between 1984 and 1999 [15], then, probably due to the selection pressure of vaccines including this subtype, there was a shift to subtype BVDV-1b [21]. In Chile as well as in Argentina BVDV-1b predominates over BVDV-1a and BVDV-1c, which are also found in that country [22].

One Uruguayan strain, 436FaUY/052014, were subtyped as BVDV-1i. This subtype is not commonly found and was never described in Argentina, Brazil and Chile. Actually, it was described for the first time in samples from United Kindom (England and Wales) in 1997 [31, 32]. This subtype has only been reported in the United Kindom where its incidence has increased in ten years from 3 % to 6 % [27].

Strain 439RvUY/082014 was subtyped as BVDV-2b as it clustered along with the references strains of this genotype named Soldan and Giessen 6 at the phylogenetic reconstruction. In Chile only BVDV-2a has been reported, while in Argentina it has been described BVDV-2a and BVDV-2b, the former with a prevalence of 3.3 % in relation to the other subtypes [21, 22]. In contrast, in Brazil BVDV-2b was the only one subtype of BVDV-2 found by Weber et al. (2014) with a prevalence of 42.4 % relative to BVDV-1. Uruguayan strain BVDV-2b is more closely genetic related with the Brazilian strains BVDV-2b LV40-30/13 and LV60-57/13 since it clustered together.

In summary, the present work revealed that BVDV seroprevalence is high in animals ranged between 6 to 12 month old and the percentage of animals having acute infections are according to those ones observed in herds having reproductive problems. For the first time we revealed the presence of BVDV subtypes BVDV-1a, BVDV-1i and BVDV-2b in Uruguayan herds. The present work aims to add new data on a very important virus for which so far there was no data on the molecular epidemiology in Uruguay. Nevertheless, more studies would be necessary in order to assess the epidemiology and molecular evolution of BVDV in Uruguayan herds. The results obtained during this research represent the first step for a better knowledge and future control programs of BVDV in Uruguay.

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Conflict of interest There is no conflict of interest in this work.

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Capítulo 2_ Manuscrito 2:

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En este trabajo se estudió la epidemiología, clínica, patología y los virus relacionados a brotes de enfermedades asociadas a BVDV.

Se describieron las siguientes presentaciones clínicas a las que BVDV se encuentra asociado:

Como resultado de la infección persistente (PI) con BVDV:

- Enfermedad de las mucosas

Como producto de la infección transitoria de BVDV y también por la co- infección con otros patógenos, se observaron infecciones:

- Digestivas
- Respiratorias
- Urinarias
- Entéricas
- Enfermedades transplacentarias
- Abortos

En estas presentaciones clínicas se identificaron a los subtipos BVDV-1a y BVDV-2b, y no se los vinculó con alguna de las presentaciones clínicas en particular.

La conclusión de este trabajo fue que BVDV contribuye a la enfermedad y muerte de bovinos en nuestro país.

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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Diseases associated with bovine viral diarrhea virus subtypes 1a and 2b in beef and dairy cattle in Uruguay

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Abstract

Bovine viral diarrhea virus (BVDV, *Pestivirus*) causes significant economic losses to the livestock industry worldwide. Although serological surveys show that BVDV exposure is widespread in cattle in Uruguay, BVDV-associated diseases are greatly underreported. The aim of this work is to describe the epidemiological, clinical, pathological, and virological findings from spontaneous outbreaks of BVDV-associated diseases in cattle in Uruguay. Diagnostic investigations were performed during 6 spontaneous disease outbreaks on beef and dairy cattle farms in the departments of Colonia, Rio Negro, and Soriano between November 2016 and April 2018. Carcasses of 8 naturally deceased cattle from these outbreaks were necropsied and subjected to histological examination and immunohistochemistry to detect BVDV antigen in the tissues. Reverse transcription real-time PCR and genomic sequencing were also performed to identify BVDV at the species and subtype levels. Other ancillary diagnostic tests, including bacterial cultures, were performed on a case-by-case basis to rule in/out differential diagnoses based on initial clinicopathological presumptive diagnoses. BVDV-associated conditions that were diagnosed in the 8 cases included mucosal disease, transient postnatal BVDV infections associated with digestive/septicemic salmonellosis by *Salmonella* serovar typhimurium, *Histophilus somni* bronchopneumonia, urinary tract coinfections with *Escherichia coli* and *Streptococcus* sp., enteric coinfection with coccidia, and transplacental fetal infections and abortions with *Neospora caninum* coinfection. BVDV-1a and BVDV-2b were each identified in four of the eight cases. We conclude that BVDV-1a and BVDV-2b contribute significantly to disease and mortality in cattle in Uruguay. Future research should estimate the economic impact of BVDV in the Uruguayan livestock sector.

Keywords Bovine viral diarrhea virus · Infectious diseases · Livestock · *Pestivirus* · South America

Introduction

Bovine viral diarrhea virus (BVDV) is an enveloped virus with a single-stranded RNA genome, which belongs to the genus *Pestivirus*, family Flaviviridae, and is distributed worldwide [1]. BVDV causes significant economic losses to the dairy and beef livestock industries, which are attributed to morbidity, mortality, growth retardation, reduced milk production, premature culling, reduced reproductive performance, and increased occurrences of other diseases [2, 3]. The two currently recognized BVDV species are BVDV-1 and BVDV-2, and they were recently renamed *Pestivirus* A and B, respectively [4, 5]. BVDV-1 is the most genetically diverse, with 21 subtypes (BVDV-1a through u) currently recognized, while 4 subtypes (a–d) have been described for BVDV-2 [6–9]. More recently, the *Pestivirus* HoBi-like virus has been proposed as a

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new BVDV species (BVDV-3 or *Pestivirus* H) based on antigenic and genetic similarities [4, 5, 10, 11].

Clinical outcomes of BVDV infection include (a) transient or acute infection with subclinical, respiratory, and/or severe digestive clinical manifestation characterized by high morbidity and variable mortality and generally associated with noncytopathic (NCP) viral strains; (b) reproductive infections, including oocyte/sperm infections that negatively affect fertility, or transplacental/congenital transmission that may result in embryonic or fetal death; mummification; abortion; congenital anomalies; stillbirths; or, if the fetus survives, the birth of persistently infected (PI) calves, particularly when the fetuses are infected by NCP strains before 4 months of gestation; and (c) mucosal disease (MD) characterized by low morbidity and very high lethality in PI animals, usually before 2 years of age. MD is associated with superinfection with a cytopathic (CP) biotype that can arise through mutation, recombination, or genomic rearrangements of the NCP viral strain that infects PI cattle [12, 13].

As viruses with worldwide distribution [9], BVDV-1 and BVDV-2 have been recognized for many years in South American countries, including Brazil [14], Argentina [15], Colombia [16], Peru, Chile [17], and Uruguay [18], while the *Pestivirus* HoBi-like virus has currently only been identified in Argentina [19] and Brazil [20] on this subcontinent. In Uruguay, the first evidence of BVDV circulation dates from 1996 [21]. A serological study revealed that BVDV exposure is widespread in beef cattle throughout Uruguay [22]. More recently, active BVDV infections and circulating species and subtypes were explored in cattle herds with reproductive problems, and BVDV-1a was revealed as the predominant species/subtype, followed by BVDV-1i and BVDV-2b [18].

Clinicopathological descriptions of BVDV-associated diseases in Uruguay and the impact of these diseases on bovine production systems in the country are lacking in the scientific literature. Recognizing and identifying these diseases in spontaneous field outbreaks is essential for establishing control programs to reduce their economic impacts at the herd and national levels. This work describes the epidemiological, clinical, pathological, and virological findings in spontaneous disease outbreaks associated with BVDV infections in cattle in Uruguay.

Materials and methods

Case selection

Eight natural cases of BVDV-associated diseases (cases 1–8) during six outbreaks (outbreaks 1–6) in commercial beef and dairy herds in Uruguay are described. Cases were diagnosed between November 2016 and April 2018 at INIA's Veterinary Diagnostic Laboratory (Animal Health Platform) in La Estanzuela, Colonia Department, Uruguay. Carcasses of the deceased cattle in cases 1–8 were provided for necropsy by veterinary practitioners and farmers. Additionally, in cases 1 and 6, serum samples collected prior to death by the veterinary practitioners were made available for testing. Epidemiological and clinical information was gathered for each outbreak when available.

Necropsy, histology, and immunohistochemistry

All 8 cattle died spontaneously at commercial farms and were subsequently necropsied. Tissue samples were collected, preserved frozen at − 20 °C for virology, and fixed in 10% neutral buffered formalin for 48 h. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4–5 μm, mounted on glass slides, and stained with hematoxylin and eosin for routine histological examination under an optic microscope (AxioScope.A1, Carl-Zeiss, Germany).

Selected formalin-fixed paraffin-embedded (FFPE) sections of various tissues from all cases were processed for immunohistochemistry (IHC) to detect *Pestivirus* antigen using a standard operating procedure kindly provided by Jan Shivers from the University of Minnesota Veterinary Diagnostic Laboratory. Briefly, heat-induced antigen retrieval was performed by placing the deparaffinized sections in a decloaking chamber (Biocare Medical) at 110 °C for 30 s. A commercially available anti-BVDV monoclonal antibody isotype IgG_{2a} produced in mice (catalogue D89, VMRD, Pullman, WA, USA) was applied as the primary antibody for 45 min at a 1:20 dilution. An anti-mouse horseradish peroxidase (HRP)–labeled polymer (EnVision+ HRP goat anti-mouse IgG, K4001, Dako) was used as the detection system, with 3-amino-9-ethylcarbazole (AEC no. 3469, Dako) as the substrate chromogen. A FFPE section of archived intestine from a naturally infected calf that tested positive for BVDV by RT-PCR and IHC was used as a positive control. For the negative controls, serial sections of all tested tissues were processed in parallel as described above, but the primary antibody was replaced with normal mouse serum (NC499L, Biocare Medical) at the same dilution.

Real-time PCR

Nucleic acids were extracted from frozen serum or tissue samples in all cases using the QIAamp® cador® Pathogen Mini Kit (QIAGEN®, Germany), following the manufacturer's instructions. Reverse transcription (RT) was performed using random primers and Super-Script II enzyme (Invitrogen®, USA). Real-time PCR assays targeting a 207-bp fragment of the 5′UTR region of the BVDV were performed using the primers BVDV190F and V326, and the Taq-Man® probe TQ-Pesti as described by Hoffman et al. [23] and Gaede et al. [24], respectively, and later modified by Maya et al.

[18], to detect BVDV-1, BVDV-2, and the HoBi-like *Pestivirus*. All real-time PCRs were performed using the SensiMixTM II Probe Kit (Bioline Reagents Ltd.) and a Rotor-Gene Q instrument (Qiagen®) following the manufacturer's recommendations.

Conventional PCR, sequencing, and phylogenetic analysis

On all real-time PCR-positive samples, a 207-bp fragment of the 5′UTR was amplified by conventional PCR, using the same primer pair used for the real-time PCR. The amplicons were sequenced at Macrogen, Inc. (Seoul, South Korea) in an ABI3730XL Genetic Analyzer (Applied Biosystems, CA, USA). The sequences were edited using SeqMan software (DNAstar Lasergene) and deposited in the GenBank database. Species and subtype assignment were done by phylogenetic analysis of Uruguayan 5′UTR sequences, along with representative BVDV-1, BVDV-2, and HoBi-like *Pestivirus* strains retrieved from the GenBank. Nucleotide sequences were aligned using Clustal W implemented in MEGA 6.06 software [25]. The model of nucleotide substitution that best fit the dataset (Kimura 2 parameters + gamma) was selected using the jModelTest program according to the Akaike Information Criterion (AIC; Akaike, 1974) [26]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method, and statistical significance Bootstrap method was carried out (1000 replicates) using Mega 6.06 version [25].

Ancillary diagnostic testing

Diagnostic investigations in each case also involved performing specific bacteriological, virological, serological, and molecular laboratory tests to assess for other pathogens, based on the presumptive clinical and pathological diagnoses in each case. Online Resource 1 summarizes the ancillary diagnostic tests performed on each of the 8 necropsied cattle.

Results

Table 1 summarizes the information on the disease diagnoses, date of diagnosis confirmation, herd geographic location, breed, production class, age, and the BVDV species/subtypes identified in all 8 cases, and whether cattle from other farms had been recently introduced to the affected herds. Additional epidemiological, clinical, and pathological information on each case/outbreak and interpretation of the ancillary and diagnostic test results (Online Resource 1) are provided below.

Outbreak 1 Outbreak 1 occurred in a herd of 340 rotationally grazing Aberdeen Angus heifers in a commercial breeding herd. Twelve heifers died sporadically from September to November 2016 (mortality 3.5%). Clinical examination of an 18-month-old heifer (case 1) revealed tachypnea (120 breaths/minute), tachycardia (48 beats/minute), a rectal temperature of 39.5 °C (normothermia), ruminal atony, foul-smelling diarrhea, dehydration, and weight loss. The animal died spontaneously after a 96-h clinical course.

The necropsy revealed multiple irregular, well-defined erosions and nonperforating ulcers up to 0.5 cm in the mucosa of the thoracic region of the esophagus (Fig. 1a), petechiae, and ecchymosis in the jejunal serosa, with brown, bloody, fluid, foul-smelling contents in the jejunum, cecum, and colon. Diffusely, the colonic mucosa was markedly reddened, with brown viscous contents and clotted blood adhered to the surface (hemorrhagic colitis) (Fig. 1b).

Microscopically, there was multifocal necrotizing ulcerative esophagitis (Fig. 1c) with microthrombosis in the submucosa and individual keratinocyte necrosis/apoptosis, multifocal superficial rumenitis and reticulitis with swelling and hydropic keratinocyte degeneration, and neutrophil transmigration in the epithelium. Severe extensive necrotizing enterotyphlocolitis with submucosal edema, microthrombosis in the lamina propria, and neutrophilic cryptitis with necrotic enterocytes was also observed. IHC for BVDV antigen detection revealed abundant, strong and frequent intralesional, finely granular, and homogeneous immunolabeling in the cytoplasm of the epithelial (keratinocytes and enterocytes) and inflammatory cells in the esophagus (Fig. 1d), small intestine, and colon.

The BVDV genome was detected in the serum via RT-qPCR (ct 27.70), and BVDV-1a was further identified by sequencing the 5′UTR genomic region (GenBank MN159214). Mucosal disease was diagnosed based on these results (Table 1), while other causes of enterocolitis in cattle, including *Salmonella* spp. and bovine coronavirus, were ruled out by specific testing (see Online Resource 1).

Outbreak 2 Outbreak 2 occurred at a dairy herd of 1222 Holstein cattle, including 580 milking cows, 40 dry cows, 200 heifers, 400 calves, and 2 bulls. Of 533 pregnant cows/heifers aged 2 to 7 years, 16% aborted between 4 and 8 months of gestation. A male fetus (case 2) that had been aborted at approximately 240 gestational days by a 5.5-year-old cow was necropsied.

The only macroscopic lesion was mild pleural petechiation in the right lung. Histology revealed multifocal moderate necrotizing lymphocytic/histiocytic encephalitis with gliosis, multifocal moderate-to-severe lymphocytic and histiocytic myocarditis, and multifocal minimal lymphocytic and histiocytic glossitis. As shown in Online Resource 1, an IHC procedure for detecting *Neospora caninum* antigen revealed intralesional immunolabeling in the fetal encephalon and heart. The protozoan's genome was detected by PCR on a

Table 1 Disease diagnosis, date, herd geographic location, breed/production class, age, BVDV species/subtype identified, and immunohistochemical results for 8 BVDV infection cases

Outbreak no./ geographic location/ date	Case no.	Disease diagnosis	Breed, production class, age	BVDV species/ subtype	BVDV IHC result	Recent cattle introductions from other farms
1/Colonia/Nov 2016	1	Mucosal disease	Aberdeen Angus heifer, beef production on pasture, 18 months old	BVDV-1a	Positive	ND
2/Rio Negro/Feb 2017	2	Abortion, acute/transient BVDV infection, coinfection with *N. caninum*	Dairy, aborted fetus, 240–270 gestational days	BVDV-1a	Negative	ND
3/Colonia/May 2017	3	Acute/transient BVDV infection, coinfection with *S. enterica* serotype typhimurium (enteric/septicemic salmonellosis)	Dairy heifer	BVDV-2b	Positive	Yes
	4	Acute/transient BVDV infection, coinfection with *H. somni* (bronchopneumonia)	Dairy heifer	BVDV-2b	Positive	
4/Colonia/June 2017	5	Acute/transient BVDV infection, urinary tract coinfection with *Streptococcus* sp. and *E. coli*	~ 2-year-old beef steer, feed lot	BVDV-1a	Positive	Yes
5/Soriano/April 2018	6	Possible mucosal disease or severe transient infection, enteric coinfection with *Eimeria* sp.	Holstein steer, beef production, 4 years	BVDV-1a	Positive	Yes
6/Colonia/Dec 2017 and Jan 2018	7	Abortion, acute/transient BVDV infection	Dairy, aborted fetus, 180–210 gestational days	BVDV-2b	Negative	ND
	8	Abortion, acute/transient BVDV infection	Dairy, aborted fetus, 240–270 gestational days	BVDV-2b	Negative	ND

IHC immunohistochemistry, *ND* not determined

frozen brain sample. Based on these results, an etiological diagnosis of encephalitis and myocarditis caused by *N. caninum* was reached. However, the BVDV genome was detected by RT-qPCR (ct 27.37) from a pool of fetal tissues and further identified as BVDV-1a (GenBank MN159211). IHC immunolabeling for BVDV antigen was negative for all fetal tissues analyzed using this technique (brain, kidney, lung, spleen, and liver), suggesting an acute/transient BVDV infection rather than a PI fetus (Table 1).

Outbreak 3 Outbreak 3 occurred on a farm dedicated to rearing dairy calves/heifers brought from various farms in different departments of Uruguay. A respiratory disease outbreak was registered in a group of approximately 200 heifers aged 3 to 4 months that had been brought to the farm approximately 30 days earlier. The respiratory disease outbreak started in 20 calves (10%) 15 days after admission, and 5 days later, approximately 80 animals (40%) were affected. Morbidity and mortality rates were 80% and 7.5% (15 heifers), respectively.

Two heifers were necropsied (cases 3 and 4). The pathological findings in case 3 included diffuse moderate fibrinous histiocytic and neutrophilic interstitial pneumonia with necrotizing bronchiolitis, multifocal neutrophilic alveolitis, and microthrombosis with multifocal pleuritis. Multifocally, the lungs and kidneys showed segmental lymphohistiocytic and necrotizing vasculitis affecting the medium-sized arterioles (Fig. 2a). Additionally, there was moderate multifocal erosive typhlocolitis with necrotizing cryptitis, with inflammatory infiltrate extended to the submucosa, along with fibrinous mesenteric lymphadenitis and moderate multifocal lymphocytic and neutrophilic abomasitis. Mild diffuse portal lymphocytic, histiocytic, and neutrophilic hepatitis with cholestasis in the bile ducts and minimal multifocal random necrotizing histiocytic and neutrophilic hepatitis throughout the parenchyma were also observed.

Salmonella enterica serotype typhimurium was isolated from the mesenteric lymph node and intestines, while bovine coronavirus and several viruses causing pneumonia were ruled out (see Online Resource 1). BVDV was identified via RT-qPCR (ct 27.35) from a pool of frozen tissues and characterized as BVDV-2b by sequencing (GenBank MN159205). IHC for BVDV antigen detection in the lung revealed finely granular, homogenous, and focal immunolabeling in the cytoplasm of smooth muscle and endothelial cells from an arteriole affected by arteriolitis and infiltrating macrophages (Fig. 2b). No immunolabeling occurred in the kidney, small intestine, colon, or liver tissues. Based on these results, we diagnosed septicemic salmonellosis in coinfection with BVDV-2b (Table 1), which was detected by IHC within the arteriolar lesions in the lung and was thus suspected to have caused the arteriolitis.

Case 4 from the same outbreak had pathological findings including severe extensive fibrinosuppurative

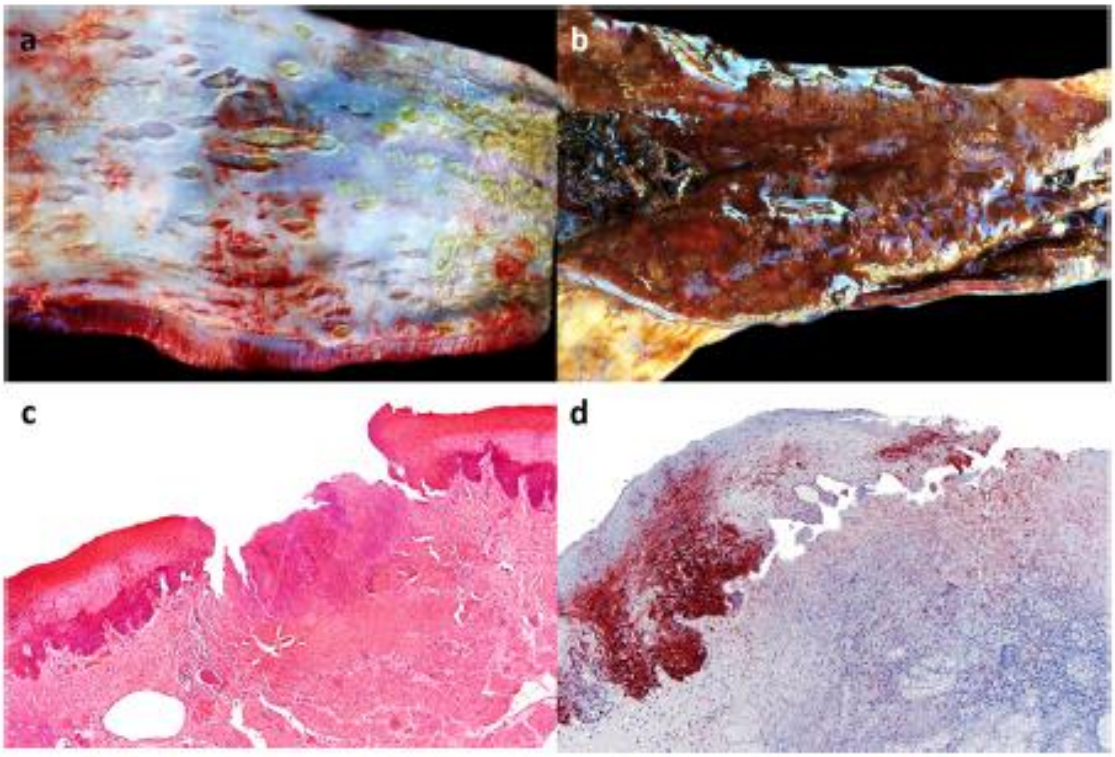

Fig. 1 Pathological findings in an Aberdeen Angus heifer with mucosal disease caused by BVDV-1a (case 1). **a** The mucosa of the esophagus shows multiple, coalescing, irregular, well-defined erosions and nonperforating ulcers (ulcerative esophagitis), some of which have a hemorrhagic halo. **b** The colonic mucosa is markedly reddened, necrotic, and ulcerated and contains scant brown-tinged fluid and few blood clots (necro-hemorrhagic colitis). **c** Low-magnification micrograph of the esophageal mucosa depicting one of the ulcers shown in **a**, with underlying necrosis of the submucosa, and hydropic degeneration of keratinocytes in the adjacent epithelium, H&E stain. **d** BVDV immunohistochemistry in a serial section of the lesion shown in **c** denoting abundant antigen immunolabeling in the esophageal mucosa adjacent to the ulcer bed

bronchopneumonia involving approximately 70% of the pulmonary parenchyma and fibrinous pleuritis. The tongue showed multifocal necrosis of the basal keratinocyte layer in the mucosa and multifocal apoptosis of individual keratinocytes in the esophagus and rumen.

Histophilus somni was isolated from the lung, and viral causes of pneumonia were ruled out (Online Resource 1). BVDV was identified in a frozen sample of pooled tissues by RT-qPCR (ct 26.08), and genome sequencing revealed the same subtype as in case 3 of this outbreak (GenBank MN159204). IHC for BVDV antigen revealed focal intralesional immunolabeling in the basal mucosal epithelium of the tongue, in the cytoplasm of the endothelial and muscular cells of blood vessels in the lungs and in macrophages infiltrating the pleura and interstitium. No immunolabeling was found in the kidney, lymph node, heart, intestinal, or rumen tissues. *H. somni* bronchopneumonia was diagnosed (Table 1) in coinfection with BVDV-2b that was detected by IHC in the pulmonary and lingual lesions.

Outbreak 4 Outbreak 4 occurred in a feedlot with approximately 300 beef cattle from different origins. An approximately 2-year-old beef steer of undetermined breed (case 5) presented inappetence and stopped drinking water. Antibiotic treatment (penicillin G procaine and dihydrostreptomycin) was administered for 5 days; however, the steer died after a 10-day clinical course. Within 6 months, two other steers with similar clinical presentations died, yielding a 1% mortality rate.

A partial necropsy was performed by the veterinary practitioner, and some organs, including the kidney, heart, liver, urinary bladder, and small and large intestines, were submitted to the diagnostic laboratory. Pathological findings in the kidneys included extensive cortical coagulative necrosis (renal infarcts) (Fig. 3a) and occasional necrotizing segmental arteriolitis with thrombosis (Fig. 3b). Severe acute/subacute transmural necrotizing, fibrinosuppurative, and hemorrhagic urocystitis with thrombosis and individual necrosis of arteriolar leiomyocytes was also present. In addition, focal acute mild colitis with necrotizing cryptitis was observed.

BVDV was detected by RT-qPCR (ct 34.04) from the frozen tissue pool and further identified as BVDV-1a via sequencing (GenBank MN159221). IHC for BVDV antigen detection showed multifocal and infrequent cytoplasmic immunolabeling in macrophages infiltrating the urinary bladder mucosa; no labeling was seen in any other analyzed tissues

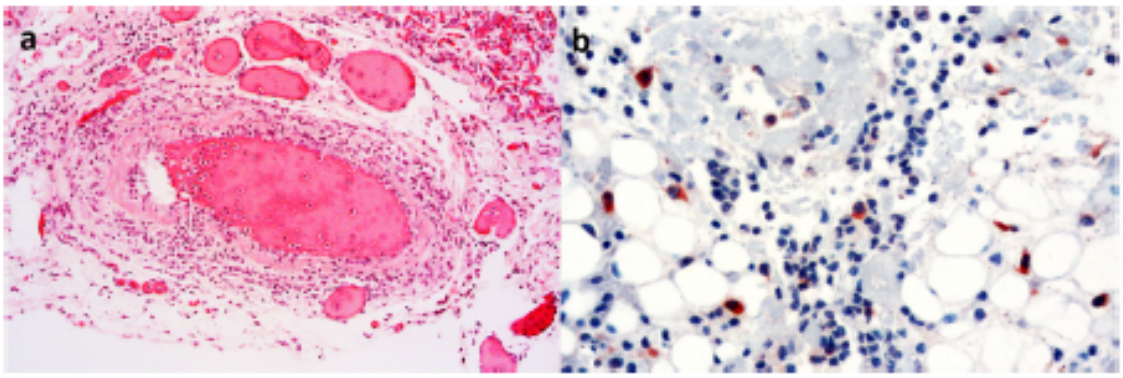

Fig. 2 Microscopic lesions in the lung of a Holstein heifer with transient BVDV-2b infection (case 3). **a** Inflammatory cells infiltrate the tunica media of a medium-sized arteriole in the pulmonary interstitium (arteriolitis), H&E stain. **b** BVDV immunohistochemistry revealing strong granular intracytoplasmic immunolabeling in macrophages infiltrating the pulmonary interstitium (pleura)

(liver, kidney, spleen, lung, and colon). Additionally, in this case, *Streptococcus* sp. was isolated from the kidney, and *Escherichia coli* was isolated from the kidney, urinary bladder, and liver (Online Resource 1). These were considered potential opportunistic secondary pathogens. Because BVDV IHC was negative in multiple tissues except the bladder, an acute/transient BVDV-1a infection was suspected.

Outbreak 5 Outbreak 5 occurred at a calf-rearing and steer-fattening operation, with 708 cattle of different breeds and origins. Between January and April 2018, 12 Holstein steers (1.7%) presented emaciation, anorexia, isolation, and diarrhea and died after a 1-month clinical course. An approximately 4-year-old Holstein steer (case 6) in poor-body condition was necropsied.

The pathological examination revealed a chronic enteropathy affecting the ileum and colon, characterized by depletion of intestinal glands/crypts in the mucosa, occasional neutrophilic/necrotizing cryptitis, and infrequent intraepithelial coccidia, morphologically compatible with *Eimeira* spp. Additionally, multifocal, moderate, chronic, lymphocytic interstitial nephritis was observed as well as moderate pleuritis with pleural fibrosis and fibrosing interstitial pneumonia and moderate suppurative lymphadenitis of undetermined etiology.

IHC for BVDV revealed strong and abundant finely granular and homogeneous intracytoplasmic immunolabeling in the epithelial cells, which was diffuse throughout all examined tissues (lung, intestines, liver, and kidney). BVDV was detected in the serum by RT-qPCR (ct 28.75), and BVDV-1a was identified by sequencing (GenBank MN186041). Other causes of enterocolitis were ruled out, including *Salmonella* spp., *Mycobacterium avium paratuberculosis*, bovine coronavirus, and group A rotavirus (Online Resource 1). Based on the overall results, this was likely either a PI calf with BVDV-1a or a severe transient infection.

Outbreak 6 Outbreak 6 occurred at a dairy farm of unknown herd size, where an increased abortion rate was registered. Two aborted Holstein fetuses (cases 7 and 8) of approximately 180–210 gestational days were necropsied. For case 7, the diagnostic investigation did not allow determining the cause of the abortion because the fetus presented no lesions, and all ancillary diagnostic tests were negative (Online Resource 1) except the RT-qPCR for BVDV (ct 32.17), which was positive from a pool of fetal tissues.

In case 8, the histological examination revealed moderate multifocal lymphohistiocytic necrotizing encephalitis, severe random multifocal necrotizing hepatitis, moderate multifocal lymphocytic myocarditis, and multifocal lymphohistiocytic placentitis with trophoblast necrosis. All lesions were highly compatible with neosporosis. *Neospora caninum* was detected intralesionally by IHC in the brain and by PCR in the same tissue (Online Resource 1). BVDV was detected by RT-qPCR (ct 33.90) from the pool of fetal tissues.

BVDV-2b was identified in both fetuses by sequencing (GenBank MN186039 and MN186040). IHC for BVDV antigen detection was negative in tissues from both fetuses, including the brain, lung, and spleen in case 7 and the brain, kidney, liver, and spleen in case 8. Based on these results, acute/transient BVDV infections, rather than PI, were suspected in both cases (Table 1).

Phylogenetic analysis

As shown in Fig. 4, the phylogenetic analysis revealed that 4 of the strains were BVDV-1a and the other 4 BVDV-2b with bootstrap values of 71% and 63%, respectively.

Discussion

The diagnoses of BVDV infection in 8 cases reported here were based on viral genome detection by RT-qPCR along with detailed pathological examinations to characterize the lesions and IHC to identify BVDV antigen in the tissues of the

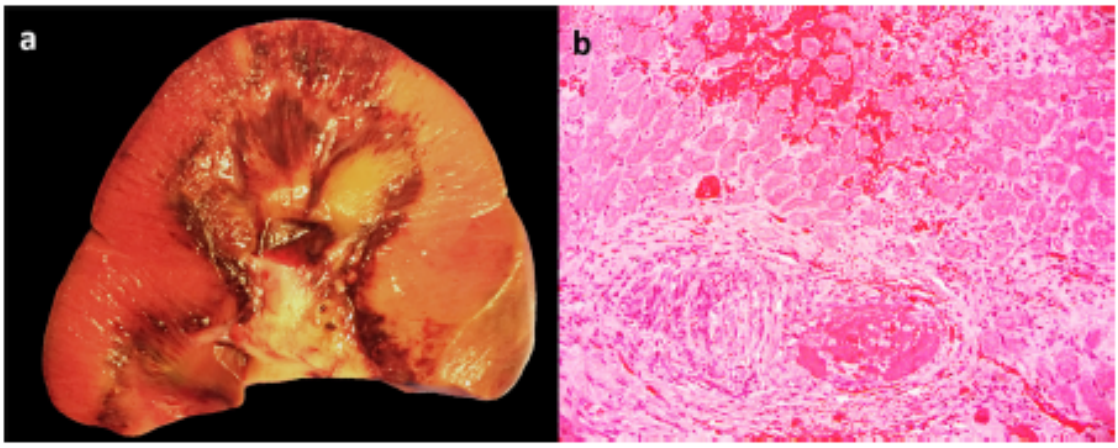

Fig. 3 Renal lesions in a beef steer infected with BVDV-1a (case 5). **a** A section of kidney shows multifocal cortical renal infarcts. **b** Micrograph of the affected kidney depicting severe arteriolitis with thrombosis (bottom) and extensive coagulative necrosis of the adjacent cortical renal tubules with interstitial hemorrhage (acute infarction), H&E stain

deceased cattle. The BVDV subtype involved in all cases was identified by sequencing the 5′UTR genomic region. Ancillary tests were performed to identify coinfections and to rule in/out differential diagnoses on a case-by-case basis (Online Resource 1). Diagnostic investigations in the 8 cases were compatible with different BVDV infection outcomes, including MD (case 1 and eventually case 6), transplacental/congenital transmission with potential transient/acute fetal infection (cases 2, 7, and 8), transient/acute postnatal infections associated with coinfections by pathogenic bacteria, such as *Salmonella* typhimurium (case 3) and *Histophilus somni* (case 4), or opportunistic bacteria, such as *Streptococcus* sp. and *E. coli* (case 5), or eventually severely transient infection associated with enteric coccidia (case 6).

The clinical signs and pathological findings in the heifer from outbreak 1 were those typically described in the literature for MD cases [13, 27]. Detection of the viral genome and the colocalization of BVDV antigen intralesionally by IHC confirmed the etiological diagnosis, while other causes of necrotizing colitis, such as *Salmonella* spp. and bovine coronavirus, were ruled out [13].

The occurrence of MD requires the presence of PI animals congenitally infected with BVDV, which are the main source of infections in the herds. PI cattle are viremic, lifelong viral shedders, and immunotolerant to the NCP strain with which they are infected because their immune systems do not recognize the BVDV antigens as foreign, thus leading to extensive viral replication in the target cells [13, 27]. In our case, viremia was confirmed by identifying BVDV-1a in the serum, while IHC allowed detecting abundant viral antigen in the alimentary tract tissues (esophagus, small, and large intestines), which showed typical MD lesions. MD occurs in PI animals when the NCP BVDV strain is converted to the cytopathic biotype via mutation, recombination, and/or genomic rearrangements. Generally, the NS2/3 viral protease containing the NCP strain is cleaved to NS2 and NS3 in the cytopathic strains [12, 28]. This results in a devastating infection that destroys the host cells, to which the animal's immune system cannot respond [13].

PI neonate calves may be clinically normal, weak, born smaller than normal, or show growth retardation; however, most die of MD before 2 years of age [12, 13], which is the same age range of the heifer with MD in case 1. The 4-year-old steer in outbreak 5 (case 6) presented severe intestinal histological lesions compatible with MD, although no macroscopic lesions typical of MD were observed in the digestive tract. This reinforces the idea that PI animals can remain asymptomatic in the herds for more than 2 years, perpetuating infection and disease. In BVDV PI cattle, IHC should show strong positive immunolabeling in all or most tissues/organs, since infection in these animals is disseminated/multisystemic, and the tissues usually contain high viral loads [29]. The strong widespread intra- and extralesional immunolabeling in the lungs, intestines, liver, and kidneys of case 6, along with the viral genome detected in the serum, indicated that this steer was viremic and most likely represented a PI animal.

Fetal responses to BVDV infection are influenced mainly by gestational age, fetal immune response, and viral biotype and subtype [29, 30]. Abortions at 6–8 gestational months identified as cases 2 and 8 in this work were most likely caused by *N. caninum* given the nature of the fetal lesions, which were typical of this protozoan, and its intralesional detection via IHC in the brain/myocardium as well as by PCR in the brain in both cases. However, coinfections with BVDV-1a (case 2) and BVDV-2b (case 8) were identified by RT-qPCR/sequencing, indicating a congenital/transplacental circulation and transmission of both viral species/subtypes in these fetuses. Because fetuses infected with NCP BVDV strains before the 4th gestational month are generally immunotolerant to

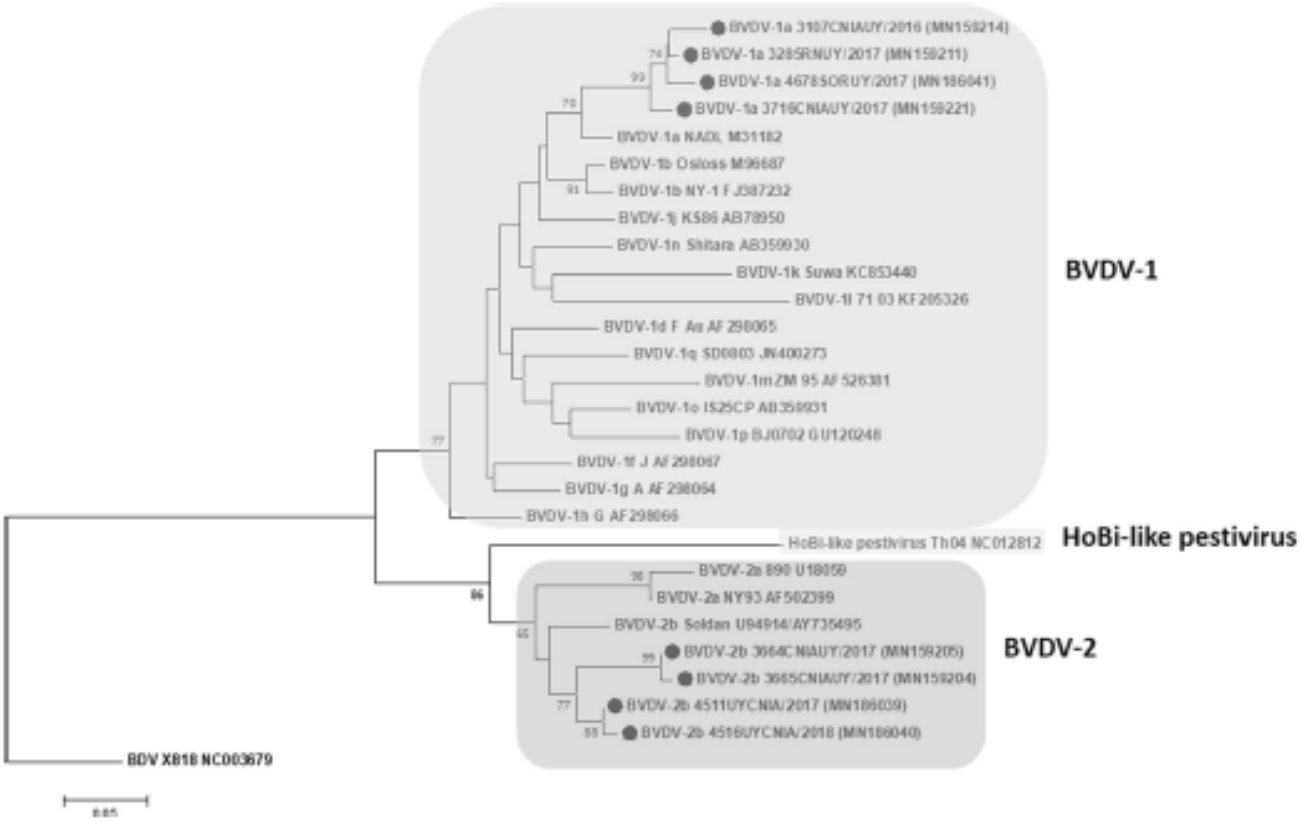

Fig. 4 Phylogenetic tree constructed by the neighbor-joining method using 207 nt of the 5'UTR of the BVDV genome. Uruguayan strains are indicated by black dots. Reference sequences of species BVDV-1, BVDV-2, and HoBi-like *Pestivirus* were retrieved from GenBank for comparison. Labels and sequence names indicating the BVDV species and subtypes. A border disease virus (BDV) sequence was included as an out-group

the virus and therefore develop persistent infections, the viral load in PI fetal tissues is usually high and detectable by IHC [29]. In contrast, in our fetuses (cases 2, 7, and 8), the virus was undetected by IHC, although it was identified by RT-qPCR, suggesting that the viral load may have been below the IHC detection limit and indirectly suggesting that these fetal infections were transient/acute. The roles of BVDV-1a and BVDV-2b infections as causes of abortions in these cases could not be determined. Interestingly, in a study conducted in dairy cattle, BVDV-induced immunosuppression was hypothesized to contribute to the occurrence of abortions due to *N. caninum* [31], although more detailed investigations are needed to confirm the possible synergistic association between these agents [32].

Fetuses that survive NCP BVDV strain infections before day 90, and more rarely up to day 125 of gestation, invariably develop immunotolerance to the virus and are born PI [33]. Immunotolerance is postulated to be mediated by the virus's ability to inhibit type I interferon production in infected fetal cells, allowing the infection to persist [34]. However, depending on the gestational age and viral strain, transplacental infection can also result in embryonic/fetal death (reabsorption, mummification, or abortion), congenital malformations, or birth of clinically healthy calves [30, 35].

BVDV subtyping of the case 2 fetus (outbreak 2) was identified as BVDV-1a, while for the case 7 and 8 fetuses (outbreak 6), subtyping was identified as BVDV-2b. Bielefeldt-Ohmann et al. [29] reported that both BVDV-2 and BVDV-1b have a similar tissue tropism, although BVDV-2 passes through the placenta more quickly. Additionally, antigenic immunolabeling in the fetal tissues was more intense for BVDV-2 than for BVDV-1b [29]. In the 3 fetuses evaluated in our study, BVDV immunolabeling was negative in all analyzed tissues, independent of the infecting viral species/subtype. This finding suggests that the fetuses were not PI, but rather were acutely/transiently infected, which may or may not have contributed to abortion due to *N. caninum* in cases 2 and 8 as discussed previously. When infection occurs in the last third of gestation, the fetal bovine immune system is sufficiently developed to respond to the virus, being comparable with a transient postnatal infection [35, 36]. Serological assays to assess whether these fetuses had developed specific humoral responses to BVDV were not performed in our study.

Transient/acute BVDV infections are largely subclinical or cause mild clinical signs, although they can induce leukopenia (lymphopenia, monocytopenia) and reduce antibody production and neutrophil release and function, resulting in immunosuppression. Such immunosuppression favors secondary bacterial and/or viral infections [13] that can contribute to respiratory, digestive, or septicemic diseases [13, 37]. In the three animals from outbreaks 3 and 4 (cases 3–5), BVDV infection likely contributed to the occurrence of pneumonic histophilosis (case 3), enteric/septicemic salmonellosis (case 4), and septicemia or urinary infection by opportunistic pathogens such as *Streptococcus* sp. and *E. coli* (case 5). These coinfections were confirmed by isolating the bacterial agents involved in each case, and their pathogenic role was established by evaluating the lesions compatible with those caused by these agents in the respective pathological examinations.

Acquiring animals without checking their BVDV infection status is an important risk factor for BVDV infection in cattle herds. This is directly related to introducing PI animals that can carry the infection or new viral strains into the herds [38–41]. In 3 of the outbreaks reported here (outbreaks 3–5), the anamnestic information revealed that cattle from different origins were introduced and mixed into the herds without prior BVDV testing, which possibly contributed to disseminating and spreading infection and disease between farms. Detecting and removing infected cattle from the herds is key in controlling BVDV infections.

BVDV-2 is generally considered more virulent, being frequently related to disease and death [36, 42]. However, in a study conducted in the USA, both BVDV-1 and BVDV-2 were diagnosed in cattle with clinical respiratory and digestive manifestations, as well as in necropsied cattle [42], with BVDV-1 being the most common species [42, 43]. BVDV-1a (cases 1, 5, and 6) and BVDV-2b (cases 3 and 4) were identified in heifers/steers in our work, indicating that both species are related to postnatal morbidity and mortality in cattle in Uruguay. In other South American countries, clinical disease has been reported for BVDV-1a, BVDV-1b, BVDV-2a, and BVDV-2b in Argentina [44–46]; BVDV-1b, BVDV-1d, BVDV-1i, and BVDV-2b in Brazil [47–50]; and BVDV-1a, BVDV-1b, and BVDV-1c in Chile and Peru [17, 51].

Although cattle exposed to BVDV in utero constitute a significant portion of clinical presentations, cases of transient/acute postnatal infection may be significant sources of direct losses [1]. Several of the deaths reported in outbreaks 3 and 4 (cases 3–5) were likely due to transient/acute BVDV infections aggravated by secondary bacterial infections. The arteriolitis observed in case 3 is a lesion frequently found in BVDV cases [13], whereas it is not a typical finding in salmonellosis [52], the agent with which this calf was coinfected. This case presented typical lesions of both agents, and although *Salmonella* typhimurium can cause disease and death by itself, it also behaves as an opportunistic pathogen, and thus, in this case, the BVDV may have predisposed to clinical salmonellosis. Additional evidence of transient/acute infection in these cases included detection of intralesional BVDV antigens by IHC in only some of the calf tissues examined by this technique.

The IHC performed in this study used a commercial monoclonal primary antibody against BVDV glycoprotein 55 (envelope glycoprotein E2) made using the BVDV NADL strain. Although this antibody binds to and cross-reacts with most BVDV strains, it does not bind to some strains (such as Oregon C24V used to produce vaccines in the USA) per the manufacturer and published information [53]. The major glycoprotein E2 is the most variable and immunodominant glycoprotein in BVDV, and as such, some strains may not bind to this antibody [54]. Our results indicated that this procedure enabled identifying at least four Uruguayan field strains: three BVDV-1a strains from outbreaks 1, 4, and 5, and one BVDV-2b strain from outbreak 3. However, additional cross-neutralization studies should be performed to assess whether this antibody cross-reacts with other local field BVDV strains, since reduced reactivity of anti-BVDV antibodies has been identified to cause failed detection of field isolates (particularly BVDV-2a) [55].

In Uruguay, a serological study conducted in beef cattle in 2000–2001 indicated that 69% of 6358 animals were seropositive and that 100% of 230 herds had at least one seropositive animal [22]. Even with a high viral circulation in the country's cattle population, only 3% of farmers implement preventive vaccinations [22]. More recently, active BVDV infections in cattle in Uruguay were explored in a study in which 390 serum samples from 14 herds were analyzed to search for viral antigens by capture ELISA and for viral genomes by RT-qPCR. Sixteen (4.1%) of these animals were positive by both techniques [18], suggesting that they were either PI animals and/or acute/transient infections acquired postnatally. Interestingly, the mortality rate was 3.5% in the herd with MD from outbreak 1 in our study, suggesting, hypothetically, that 3.5% of this herd could have been PI animals, a value close to that found by Maya et al. [18]. Although the prevalence of PI animals in a herd is generally < 2%, it can be as high as 25–30% in herds where many cows/heifers were exposed to NCP BVDV strains during early pregnancy [13].

The only published study to explore BVDV genetic diversity in Uruguay found that the main species/subtype was BVDV-1a [18]. Coincidentally, this subtype was found in the MD case (outbreak 1) and in outbreaks 2, 4, and 5 described herein, indicating that, besides being frequent, this subtype is associated with disease and death in cattle, which is unprecedented in Uruguay in the scientific literature. Based on our results, BVDV-2b can also be regarded as a significant cause of disease in Uruguay. In addition, this work expands the geographical distribution (south) and the biotype of

infected cattle (dairy) in Uruguay for BVDV-2b, since this subtype had previously been detected only in beef cattle in the department of Rivera, in northern Uruguay [18].

Vaccines commercially available for BVDV prevention in Uruguay are manufactured with inactivated virus and can only provide partial protection, unlike modified-live vaccines used in other regions [56], including South American countries such as Brazil [57]. Recent investigations in Argentina have led to developing an enhanced BVDV subunit vaccine expressed in a baculovirus, based on a truncated E2 glycoprotein fused to a single-chain antibody that targets antigen-presenting cells [58]. This vaccine's immunogenicity was tested in guinea pigs and cattle, and immunized cattle developed high levels of neutralizing antibodies against BVDV up to 1 year after immunization. Based on these results, the vaccine was scaled up and registered and is being commercialized as the first Argentinean subunit vaccine for cattle [59]. Whether these vaccines protect against Uruguayan BVDV strains remains to be addressed.

Conclusions

BVDV causes pathology and is associated with diseases and mortality in beef and dairy cattle in Uruguay. More extensive and systematic studies are necessary to determine the epidemiology and economic impacts of BVDV for livestock in Uruguay as well as the distribution and frequency of the different species/subtypes involved in clinical settings. Cost-benefit analyses and feasibility studies could help establish preventive and control programs for BVDV at the farm, regional, and national levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Capítulo 3_ Manuscrito 3:

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En este trabajo se analizaron 2546 muestras colectadas entre 2015 y 2017, y se analizaron por PCR y secuenciación los fragmentos genómicos 5´UTR/N^{pro}, N^{pro} y la glicoproteína de membrana E2.

Los principales hallazgos de este trabajo fueron los siguientes:

- La epidemiología molecular descrita previamente en Maya et al., (2016) continúa incambiada. Es decir, circulan los subtipos BVDV-1a, 1i, y BVDV-2b, y el subtipo BVDV-1a continúa siendo el más prevalente de acuerdo con nuestro muestreo.
- Se realizaron estudios de dinámica poblacional y revelaron que BVDV-1a estaría circulando en nuestros rodeos desde 1990 aproximadamente.
- BVDV-1a se diseminó y evolucionó acumulando mutaciones puntuales a una tasa de 3,48 × 10−3 sustituciones / sitio / año, adquiriendo características genéticas específicas que dieron lugar a dos linajes genéticos uruguayos de BVDV-1a
- Estos linajes uruguayos difieren de las cepas BVDV-1a que circulan en otros países y de la cepa de vacuna que se utiliza actualmente en Uruguay.
- Las diferencias más notables entre las cepas de campo y de vacuna se encontraron en la glicoproteína E2, lo que sugiere que las sustituciones de aminoácidos podrían resultar en un fallo de la protección cruzada / neutralización después de la vacunación.
- Este es el primer estudio que compara cepas de campo y vacunas de BVDV uruguayo con otras cepas de BVDV de todo el mundo.
- Los resultados obtenidos en este estudio serán de gran utilidad para desarrollar un programa de inmunización adecuado para el BVDV en Uruguay mediante la identificación de cepas de campo locales como candidatas para el desarrollo de vacunas.

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ORIGINAL ARTICLE

An extensive field study reveals the circulation of new genetic variants of subtype 1a of bovine viral diarrhea virus in Uruguay

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Abstract

Bovine viral diarrhea virus (BVDV) is a major pathogen worldwide, causing significant economic losses to the livestock sector. In Uruguay, BVDV seroprevalence at the farm level is >80%. In this work, 2546 serum, blood or tissue samples collected from animals suspected of being affected by BVD between 2015 and 2017 were analyzed by reverse transcription PCR and sequencing. Analysis of the BVDV genomic regions 5'UTR/N^{pro}, N^{pro} and E2 revealed that BVDV-1a, 1i and 2b circulate in the country, with BVDV-1a being the most prevalent subtype. Population dynamics studies revealed that BVDV-1a has been circulating in our herds since ~1990. This subtype began to spread and evolve, accumulating point mutations at a rate of 3.48×10^{-3} substitutions/site/year, acquiring specific genetic characteristics that gave rise to two local genetic lineages of BVDV-1a. These lineages are divergent from those circulating worldwide, as well as the vaccine strain currently used in Uruguay. The most notable differences between field and vaccine strains were found in the E2 glycoprotein, suggesting that the amino acid substitutions could result in failure of cross-protection/neutralization after vaccination. This is the first study that compares Uruguayan BVDV field and vaccine strains with other BVDV strains from throughout the world. The results obtained in this study will be very useful for developing a suitable immunization program for BVDV in Uruguay by identifying local field strains as candidates for vaccine development.

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Introduction

Bovine viral diarrhea (BVD) is a disease of economic importance worldwide that causes a wide variety of clinical signs and reproductive failure in cattle [1]. The etiological agent, bovine viral diarrhea virus (BVDV), induces reproductive disorders (embryonic death, abortion, reduced fertility), immunosuppression, respiratory problems, diarrhea, and persistently infected (PI) calves that are immunotolerant to the virus [1–3]. According to a review by Yarnall and Thrusfield published in 2017, the economic impact of BVDV can vary between 0 to 710 dollars per cow per year. This range considers the subclinical disease, generally observed in herds with endemic BVDV infection, as well as epidemic or acute clinical infections that are seen more frequently in naive herds [4].

BVDV belongs to the genus *Pestivirus* in the family *Flaviviridae*, with a single-stranded positive-sense RNA genome of approximately 12.3 kb in length flanked at both ends by untranslated regions (UTRs). The only open reading frame (ORF) of BVDV encodes a single polyprotein that is cleaved co- and post-translationally into 11-12 structural

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and non-structural proteins, including N^{pro}, C, Erns, E1, E2, p7, NS3 (NS2-NS3), NS4A, NS4B, NS5A, and NS5B [5].

There are three BVDV species currently recognized by the International Committee of Taxonomy Virus (ICTV), namely *Pestivirus A*, *B* and *H*, which include BVDV-1, BVDV-2, and HoBi-like pestivirus, respectively [6, 7].

Previous studies using BVDV nucleotide sequences of the 5'UTR, N^{pro} and E2 genomic regions have revealed that BVDV-1 was the first to emerge, around 1802, followed by HoBi-like pestivirus in 1880 and BVDV-2 in 1890 [7].

BVDV-1 is the most genetically diverse, and at least 21 subtypes (BVDV-1a to BVDV-1u) have been described [8]. The greatest diversity of BVDV-1 subtypes has been described in strains circulating in Europe, China and Turkey [9]. In contrast, for BVDV-2, only four subtypes (BVDV-2a to BVDV-2d) have been described, and subtype 2a seems to predominate on all continents [9–13].

To study the genetic diversity of BVDV, different genomic regions have been used, including the 5'UTR, the membrane glycoprotein E2, the autoprotease N^{pro}, and the protease NS3 [10, 14–16]. Currently, the 5'UTR is the main genomic region used for this purpose. Analysis of this genomic region has allowed the demarcation of BVDV species. However, due to its short length and high nucleotide conservation level, BVDV-1 subtypes usually cannot be easily defined, sometimes leading to incorrect subtype assignment [17, 18]. The use of 5'UTR/N^{pro} allows proper assignment of BVDV strains to the different species and subtypes of BVDV [19]. Alternatively, the E2 glycoprotein, which is 1122 nucleotides in length, has been also used for this purpose. E2 is a less-conserved genomic region, and the most variable region of E2 is the amino-terminal end. The DA and DB domains of the E2 protein (residues 4-87 and 88-164, respectively), are the most exposed on the virus surface and therefore contain the main antigenic sites of the virion [20, 21]. Due to its variability, phylogenetic studies carried out with E2 have shown higher resolution than those based on other genomic regions. Additionally, comparative analysis of field and vaccine strains has been useful for discovering strains that are capable of escaping neutralizing antibodies [22].

BVDV-1 has a global distribution, while BVDV-2 seems to have a more restricted geographic distribution [9]. In South America, BVDV-1 predominates, with BVDV-1b being the most frequent subtype [9]. BVDV-1a is the most abundant subtype in Brazil, and it predominated in Argentina from 1984 to 1999 [23, 24]. There is a common belief that, due to the selective pressure produced by the BVDV-1a vaccine, there was a shift towards subtype BVDV-1b. Currently, BVDV-1b appears to be the most prevalent subtype in Argentina and is currently diversifying into a new group named "Arg1b" [25].

In our previous work, we reported that BVDV-1 and BVDV-2 circulate in Uruguayan herds, with BVDV-1a being the most frequent subtype [19]. The study of genetic variability among Uruguayan strains provides information to improve the diagnosis of the disease and represents a starting point for identifying local field strains for development of protective vaccines using homologous strains. In this work, as part of a national animal health project, we conducted a broad study of the molecular epidemiology of BVDV in Uruguay and deeper evolutionary analysis of BVDV-1a strains, the most prevalent subtype.

The results of this work will help to better understand the epidemiology, evolution and genetic variability of Uruguayan strains of BVDV and will provide tools to strengthen prevention and control strategies.

Materials and methods

Uruguayan BVDV sequences

Sample collection

From March 2015 to December 2017, a total of 2546 serum, blood and tissue samples were collected at the "Plataforma de Salud Animal" of the "Instituto Nacional de Investigación Agropecuaria". Samples were obtained from calves with and without diarrhea, aborted fetuses, and heifers from farms where abortions had been reported. Samples were submitted to the molecular virology laboratory at the "Centro Universitario Salto, Universidad de la República", and stored at -20 °C until testing.

RNA extraction, reverse transcription and sample screening by real-time PCR

Viral RNA was extracted from all samples using a QIAamp cador Pathogen Mini Kit (QIAGEN, Germany) following the instructions specified by the manufacturer. In blood and serum samples, the protocol "Purification of pathogenic nucleic acids from fluid samples" was followed. Tissue samples were pretreated according to the "Pretreatment T1" protocol as described in the instructions. Reverse transcription was carried out using random primers and SuperScript II enzyme (Invitrogen, USA). For the detection of BVDV-1, BVDV-2 and HoBi-like pestivirus, a real-time PCR assay targeting 207 bp of the 5'UTR region of BVDV was performed using primers BVDV190F and V326 and the TaqMan® probe TQ-Pesti as described by Hoffman et al. and Gaede et al., respectively, and latter modified by Maya et al. [19, 26, 27]. All real-time PCR reactions were performed using a SensiMix II Probe Kit (Bioline Reagents Ltd.) and a Rotor-Gene Q instrument (QIAGEN) following the manufacturer's recommendations.

Amplification by conventional PCR

A fragment of 207 bp of the 5'UTR was amplified from real-time-PCR-positive samples, using the same primer pair that was used for the real-time PCR. Furthermore, a fragment of 428 bp of the N^{pro} genomic region was amplified as described by Maya et al. [19]. A third amplicon of 606 bp belonging to the carboxyl-terminal region of the E1 glycoprotein (215 bp) and the amino-terminal region of the glycoprotein E2 (391 bp) was amplified using primers B11 and B32 with PCR conditions described by Couvreur et al. [28]. The resulting amplicons were sequenced by Macrogen (Seoul, South Korea) in an ABI3730XL Genetic Analyzer (Applied Biosystems, CA, USA).

Phylogenetic analysis

All of the sequences were edited using SeqMan Software (DNASTAR Lasergene) and aligned using the Clustal W algorithm in MEGA version 6.06 [29]. Genotype assignment was done by phylogenetic analysis of Uruguayan 5'UTR and N^{pro} sequences concatenated into a fragment of 607 bp (5'UTR and N^{pro} sequences overlap by 28 bp) and named "5'UTR/N^{pro}", along with sequences from Uruguayan BVDV strains published by Maya et al. (n = 14) and those of representative BVDV-1, BVDV-2 and HoBi-like pestivirus strains retrieved from the GenBank database. Sequences from Uruguayan strains are listed in Table 1. For samples from which only the 5'UTR region could be amplified, the subtype was determined using the NCBI BLAST tool [30].

Analysis of the genetic variability of BVDV-1a subtypes was performed using the coding region of N^{pro} sequences (410 bp) and the amino-terminal E2 nucleotide sequences (391 bp) from Uruguayan strains and BVDV-1a strains from other countries based on the review by Yesilbag et al. [9], using sequences available in the GenBank database. The sequences used in these studies are summarized in Online Resources 1 and 2.

In all phylogenetic analyses, a reference sequence of border disease virus (BDV) was used as out-group. The model of nucleotide substitution that best fit the different datasets (GTR + gamma) was selected using the jModelTest program according to the Akaike information criterion (AIC; Akaike, 1974) [31]. Phylogenetic trees were constructed by the maximum-likelihood (ML) method, and statistical significance was evaluated by the bootstrap method (1,000 replicates) in MEGA version 6.06 [29].

Sequence analysis of Uruguayan BVDV-1a strains at the nucleotide and amino acid levels

The genetic distances of nucleotide and amino acid sequences were determined for the Uruguayan BVDV-1a strains and between the Uruguayan BVDV-1a strains and the NADL strain using N^{pro} sequences (410 bp, and 136 amino acids) and those of E2 (391 bp and 131 amino acids), using MEGA version 6.06 [29].

The amino acid substitutions found in glycoprotein E2 of the Uruguayan BVDV-1a strains and the NADL strain were mapped to the crystallographic structure of E2 determined by El Omari et al. [20] (PDB ID: 2Y2Q) using PyMOL [32].

Population dynamics analysis of Uruguayan BVDV-1a

Before performing molecular clock analysis, the TempEst program was used to determine whether the sequences used in this work showed a temporal structure [33]. The time of the most recent common ancestor (tMRCA), the evolutionary rate of the 5'UTR/N^{pro} (607 bp) of the Uruguayan BVDV-1a strains, and their demographic history were jointly estimated using the BEAST v1.7.5 package (available online: http://tree.bio.ed.ac.uk/software/beast/) [34]. Bayesian analysis was conducted under relaxed molecular clock models with uncorrelated lognormal (UCLN) and exponential (UCED) distributions and the strict molecular clock model. The three molecular clock models were evaluated in combination with the constant size, exponential growth, expansion growth and Bayesian skyline coalescent tree prior using Bayes Factors. Markov chain Monte Carlo (MCMC) simulations were run for 200 million generations, and the results were visualized using the Tracer v1.5.0 program (available from http://beast.bio.ed.ac.uk/Tracer), excluding the initial 10% of the run as burn-in. The effective sample size (ESS) values were checked to evaluate the convergence of the analysis, accepting only values higher than 200 for all of the parameters. The effective number of infections was represented graphically using Tracer v1.5.0.

Results

Genetic diversity of Uruguayan BVDV strains based on analysis of 5'UTR/N^{pro} sequences

Twenty-three samples were positive for BVDV by real-time PCR. These 23 samples, added to the 16 samples previously obtained by our group, gave a total of 39 samples that were positive for BVDV (Table 1) [19].

In five of the 23 samples, only the 5'UTR region could be amplified, and the subtype was determined using the NCBI BLAST tool, with three samples being characterized as BVDV-1a, and two samples as BVDV-2b. In our previous work, two of the 16 positive samples were classified as BVDV-1a by BLAST, so we obtained a total of five BVDV-1a and two BVDV-2b isolates (Table 1) [19].

Table 1 Sample name, including the date of collection, geographic location, GenBank accession numbers for the 5'UTR/N^{pro}, 5'UTR and E1-E2 sequences, and subtype designation of the Uruguayan strains from this study and those reported by Maya et al. (2016)

Sample name	Geographic location (department)	Accession number 5'UTR/N^{pro}	Accession number 5'UTR	Accession number E1-E2	BVDV subtype
408TboUY/072014	Tacuarembó	KT833787		MN159231	BVDV-1a
409TboUY/072014	Tacuarembó	KT833788		MN159238	BVDV-1a
429TboUY/082014	Tacuarembó	KT833789			BVDV-1a
430TboUY/082014	Tacuarembó	KT833790		MN159230	BVDV-1a
431TboUY/082014	Tacuarembó	KT833791			BVDV-1a
432TboUY/082014	Tacuarembó	KT833792		MN159229	BVDV-1a
433FaUY/032014	Florida	KT833793		MN159228	BVDV-1a
434FaUY/032014	Florida	KT833784		MN159227	BVDV-1a
435FaUY/032014	Florida	KT833794		MN159226	BVDV-1a
437TboUY/042014	Tacuarembó	KT833796			BVDV-1a
438TboUY/042014	Tacuarembó	KT833797		MN159236	BVDV-1a
653TboUY/082014	Tacuarembó	KT833798			BVDV-1a
651TboUY/082014	Tacuarembó		KT833785		BVDV-1a
652TboUY/082014	Tacuarembó		KT833786		BVDV-1a
588SaUY/2015	Salto	MN159206		MN159225	BVDV-1a
754UYAFA4/112015	Unknown	MN159207		MN159224	BVDV-1a
1284TyTUY/022016	Treinta y Tres	MN159208			BVDV-1a
1532SJUY/042016	San José	MN159209			BVDV-1a
2144UY/2016	Unknown	MN159220			BVDV-1a
2145UY/2016	Unknown	MN159219			BVDV-1a
2146UY/2016	Unknown	MN159218			BVDV-1a
2147UY/2016	Unknown	MN159217		MN159235	BVDV-1a
2148UY/2016	Unknown	MN159213			BVDV-1a
2402SJUY/2016	San José	MN159215		MN159234	BVDV-1a
2405SJUY/2016	San José	MN159216		MN159233	BVDV-1a
2514SJUY/2016	San José	MN159210		MN159232	BVDV-1a
3107CNIAUY/2016	Colonia	MN159214		MN159237	BVDV-1a
3285RNUY/2017	Río Negro	MN159211			BVDV-1a
3387CNESUY/2017	Canelones	MN159212			BVDV-1a
3397CNESUY/2017	Canelones		MN159203		BVDV-1a
3716CNIAUY/2017	Colonia	MN159221			BVDV-1a
3723CNIAUY/2017	Colonia		MN159202		BVDV-1a
3738LAVUY/2017	Lavalleja		MN159201		BVDV-1a
436FaUY/052014	Florida	KT833795			BVDV-1i
439RvUY/082014	Rivera	KT833799			BVDV-2b
2391UYRN/2016	Río Negro	MN159223			BVDV-2b
2769RNUY/2016	Río Negro	MN159222			BVDV-2b
3664CNIAUY/2017	Colonia		MN159205		BVDV-2b
3665CNIAUY/2017	Colonia		MN159204		BVDV-2b

From the remaining 18 positive samples, the 5'UTR and N^{pro} genomic regions could be successfully amplified, sequenced, edited and subsequently concatenated to 5'UTR/N^{pro} fragments. Adding these 18 sequences to those described by Maya et al. [19] (n = 14), a total of 32 Uruguayan 5'UTR/N^{pro} sequences from strains from 2014-2017 were used for phylogenetic analysis and subsequent studies.

According to the results obtained from jModelTest, the best model of nucleotide substitution that described our dataset was GTR + gamma.

The 32 samples were genotyped by performing a phylogenetic analysis based on a 607-bp fragment of the 5'UTR/N^{pro} region (Fig. 1). We found that 29 Uruguayan strains belonged to the species *Pestivirus A* (BVDV-1), with 91% statistical support, and three belonged to the species

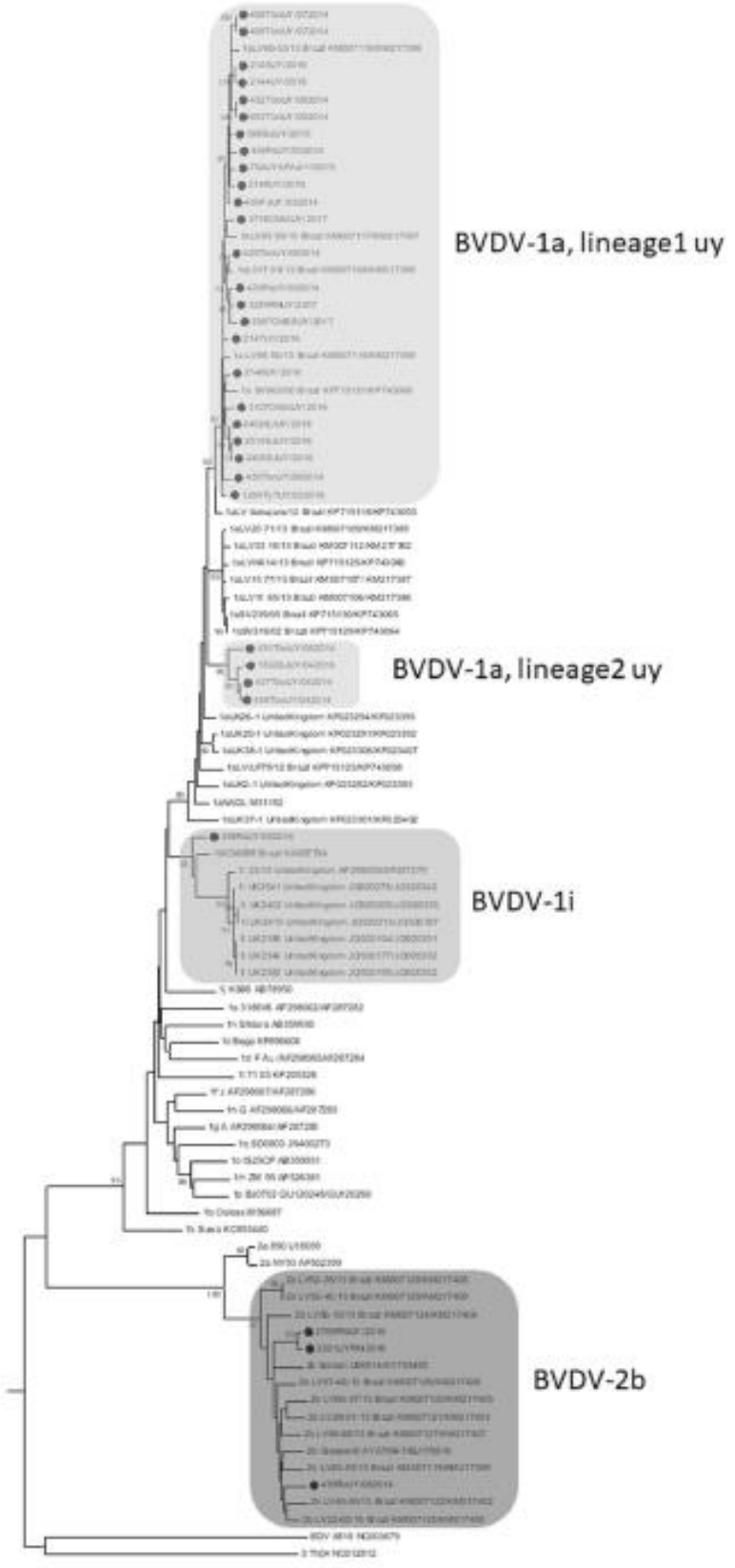

Fig. 1 Phylogenetic analysis of the 5'UTR/N^{pro} region of BVDV strains. Uruguayan strains are indicated by black dots. BVDV-1a, 1i and 2b are highlighted in gray and indicated on the right side of the figure. Numbers at the branches of the trees are bootstrap values. A BDV (border disease virus) sequence was included in the analysis as an out-group

Pestivirus B (BVDV-2), with 100% support. Inside the BVDV-1 cluster, 28 of the 29 samples belonged to the subtype BVDV-1a, and one sample belonged to the subtype BVDV-1i. All BVDV-2 strains were assigned to BVDV-2b subtype.

The sample name, viral species/subtype, geographic location (department) and GenBank accession number of the 5 'UTR/N^{pro}, 5'UTR and E1-E2 genomic regions are summarized in Table 1.

Phylogenetic analysis shows that the Uruguayan BVDV-1a strains (n = 28) were subdivided into two clades, named BVDV-1a lineage 1 UY and BVDV-1a lineage 2 UY (Figure 1). BVDV-1a lineage 1 UY was the main clade, constituted by 24 BVDV-1a sequences. This lineage formed a group supported by an 81% bootstrap value, along with five Brazilian strains (LV60-53/13, LV85-59/13, LV17-09/13, SV663/00 and LV86-80/13).

The smaller Uruguayan subgroup, designated as BVDV-1a lineage 2 UY, with 99% bootstrap support, is formed by the remaining four Uruguayan sequences (see Fig. 1).

The Uruguayan strain 436FaUY/052014 of the BVDV-1i subtype clusters together with the Brazilian strain ACM/BR/2016 and with strains from the United Kingdom. Within this group, the strains from the United Kingdom grouped separately from the two Latin American strains, with 99% statistical support.

The three Uruguayan strains of the BVDV-2b subtype were divided into two groups: strain 439RvUY/082014 grouped with Brazilian strains of this subtype, and strains 2391UYRN/2016 and 2769UYRN/2016 together formed a separate group supported by a bootstrap value of 99%.

Analysis of the N^{pro} genomic region

Nucleotide and amino acid distances among Uruguayan BVDV-1a strains

The 24 sequences of BVDV-1a lineage1 UY had a nucleotide sequence divergence of 4.4% (18/410) and an amino acid sequence divergence of 2.9% (4/136). The 4 sequences of BVDV-1a lineage 2 UY had a nucleotide sequence divergence of 2.9% (12/410) an amino acid sequence divergence of 0.7% (1/136). These two Uruguayan lineages diverge in 11.5% of their nucleotides (47/410), and 5.2% (7/136) of their amino acids (Table 2).

Both Uruguayan lineages are genetically divergent with respect to the NADL vaccine strain. BVDV-1a lineage 1 UY and the NADL strain diverge in 11% of their nucleotides (45/410) and 5.1% of their amino acids (7/136). The BVDV-1a lineage 2 UY diverges from the NADL strain in 9.2% of its nucleotides (38/410) and 5.9% of its amino acids (8/136) (Table 2). In the N^{pro} protein, the two Uruguayan lineages have the residues $N/E_{30}I_{39}K_{75}F_{110}$, which differ from those in the NADL strain, which has $D_{30}V_{39}R_{75}S_{110}$ (Table 3a).

Table 2 Nucleotide and amino acid sequence differences between Uruguayan BVDV-1a lineage 1 and 2 and the NADL strain

N^{pro}	Nucleotide:amino acid (number of differences)		
	BVDV-1a lineage 1 UY	BVDV-1a lineage 2 UY	NADL
BVDV-1a lineage1 UY	18:4	48:7	45:7
BVDV-1a lineage2 UY	48:7	12:1	38:8
E2	Nucleotide:amino acid (number of differences)		
	BVDV-1a lineage 1 UY	BVDV-1a lineage 2 UY	NADL
BVDV-1a lineage1 UY	33:12	86:26	83:31
BVDV-1a lineage2 UY	86:26	—	89:27

Table 3 Amino acid sites with differences between BVDV-1a lineage 1 UY, BVDV-1a lineage 2 UY and the NADL strain **a)** in the sequence of the N^{pro} protein and **b)** in glycoprotein E2. The amino acids that are part of the E2 fingerprint of BVDV-1a lineage 1 UY are shown in bold

a)	Strain/residue in N^{pro}	30	39	75	110
	BVDV-1a lineage1 UY	N/E	I	K	F
	BVDV-1a lineage2 UY	N	I	K	F
	NADL	D	V	R	S

b)	Strain/residue in E2	15	20	**38**	49	**54**	**68**	71	**81**	87	88	90
	BVDV-1a lineage1 UY	S	P	**T**	K	**V**	**T**	S/T	**E**	L/Q/S	R	G
	BVDV-1a lineage2 UY	D	P	M	K	M	I	S	K	Q	R	E
	NADL	D	Q	K	E	M	I	T	K	R	K	E

Phylogenetic analysis of the N^{pro} genomic region

A second phylogenetic analysis (Fig. 2) was performed with 143 nucleotide sequences of the N^{pro} protease (410 bp) from the BVDV-1a subtype isolates from Uruguay (n = 28) and strains from Austria, Brazil, China, Canada, the United Kingdom and the United States of America, (Online resource 1) selected based on a review by Yesilbag et al. [9].

The results of these studies revealed that several groups of BVDV-1a strains can be observed (Fig. 2). The Uruguayan strains of lineage BVDV-1a showed the same topology when using the 5'UTR/N^{pro} or N^{pro} region for analysis (see Fig. 1 and 2). Strains of BVDV-1a lineage 1 UY were grouped with 92% statistical support with the five Brazilian strains and the Chinese strain G5S. Strains of BVDV-1a lineage 2 UY grouped together with a bootstrap value of 88%. Strains from the United Kingdom formed several groups (named UK1 to UK8). The Brazilian strains were separate from Uruguayan lineage 1 UY and formed a group (BR clade). The same was observed for the Canadian strains, which formed a group called the "CA clade" (Online Resource 1).

Population dynamics analysis of Uruguayan BVDV-1a isolates

To better characterize the Uruguayan BVDV1a strains, and their evolutionary and demographic history, we estimated their tMRCA and molecular clock. The nucleotide substitution model that best fit our data was GTR + gamma. The results obtained using Tempest showed that the 5'UTR/N^{pro} genomic region had a temporal structure, and therefore we could proceed to other estimates.

The results of these analyses revealed that this subtype began to spread in Uruguay in 1990 at a substitution rate of 3.48×10^{-3} substitutions/site/year (Table 4).

Analysis of the E1-E2 genomic region

Nucleotide and amino acid distances between Uruguayan BVDV-1a strains

E1-E2 nucleotide sequences (603 bp) were obtained for 15 Uruguayan strains of the BVDV-1a subtype, 14 of BVDV-1a lineage 1 UY, and one of the BVDV-1a lineage 2 UY (438TboUY/042014), (see Table 1). The amplified region of E2 (391 bp, and 131 amino acids of a total of 373) is shown in white in Fig. 4a and b and corresponds to domains DA (amino acids 4-87) and partly to domain DB (amino acids 88-164).

The E1-E2 region of the BVDV-1a lineage 1 UY isolates showed a genetic diversity of 5.5% at the nucleotide level and 6% at the amino acid level (Table 2). BVDV-1a lineage 1 UY differed from BVDV-1a lineage 2 UY in 14.3% (86/603) of its nucleotides and 12.9% of its amino acids (Table 2). BVDV-1a lineage 1 UY has a nucleotide divergence from the NADL vaccine strain of 13.8%, and 15.4% at the amino acid level. When BVDV-1a lineage 2 UY (strain 438TboUY/042014) and the NADL strain were compared, a divergence of 14.8% and 13.4% at the nucleotide and amino acid level, respectively, was observed (Table 2).

The NADL strain differs from the two Uruguayan lineages of BVDV-1a at 11 amino acid positions in the E2 glycoprotein (Table 3b). Seven amino acid changes were found in beta sheets (aa 15, 20, 38, 49, 54, 68 and 81) and are shown as white spheres in Fig. 4b, and the four remaining residues were in the loops between the beta sheets (aa 71, 87, 88 and 90) and are shown as light gray spheres in Fig. 4b. El Omari et al. mapped a linear epitope of CSFV in the structure of glycoprotein E2 of BVDV from amino acid 83 to 90 in the DA and DB domains, in a loop between two beta sheets (light gray, Fig. 4a) [20]. Substitutions at amino acids 87, 88 and 90 found in Uruguayan lineages of BVDV-1a with respect to the NADL strain are part of this linear epitope (represented by light gray spheres in Fig. 4b). An immunodominant epitope has been mapped in BVDV E2 from amino acid 71 to 74 of E2 (light gray, Fig. 4a) [35]. A substitution at amino acid 71 of E2 was observed in most of the Uruguayan BVDV-1a strains (12 of 15) when compared to the NADL strain (represented by a dark gray sphere in Fig. 4b).

Uruguayan BVDV-1a lineages differ from each other by six non-synonymous substitutions in the E2 protein (aa 15, 38, 54, 68, 81 and 90), and substitutions at positions 38, 54, 68 and 81 of E2 were found only in the Uruguayan strains of BVDV-1a lineage 1 UY (Table 3b).

Phylogenetic analysis of the E2 genomic region

A third phylogenetic analysis was done using 33 nucleotide sequences from the amino-terminal region of the membrane glycoprotein E2 (391 bp) (see Fig. 3), including strains from the BVDV-1a subtype from Uruguay (n = 15) and strains from China, Japan, the United States of America and Argentina (Online Resource 2), selected on the basis of a review by Yesilbag et al. [9]. To date, no available and comparable sequences of the amino-terminal region of E2 from Austria, Brazil and the United Kingdom are available, so a phylogenetic tree of N^{pro} and E2 could not be made with sequences from those countries. The Canadian sequences of E2 obtained by Chernick et al. [22] were too short (245 bp) to be included in the analysis. The results of these studies are shown in Figure 3.

Strains of BVDV-1a lineage 1 UY clustered together with the Chinese strain G5S, with 95% bootstrap support, while BVDV-1a lineage 2 UY grouped with the Chinese strain GS24, with 94% bootstrap support (see Fig. 3). The BVDV-1a strains from Japan formed a separate group called the "JP

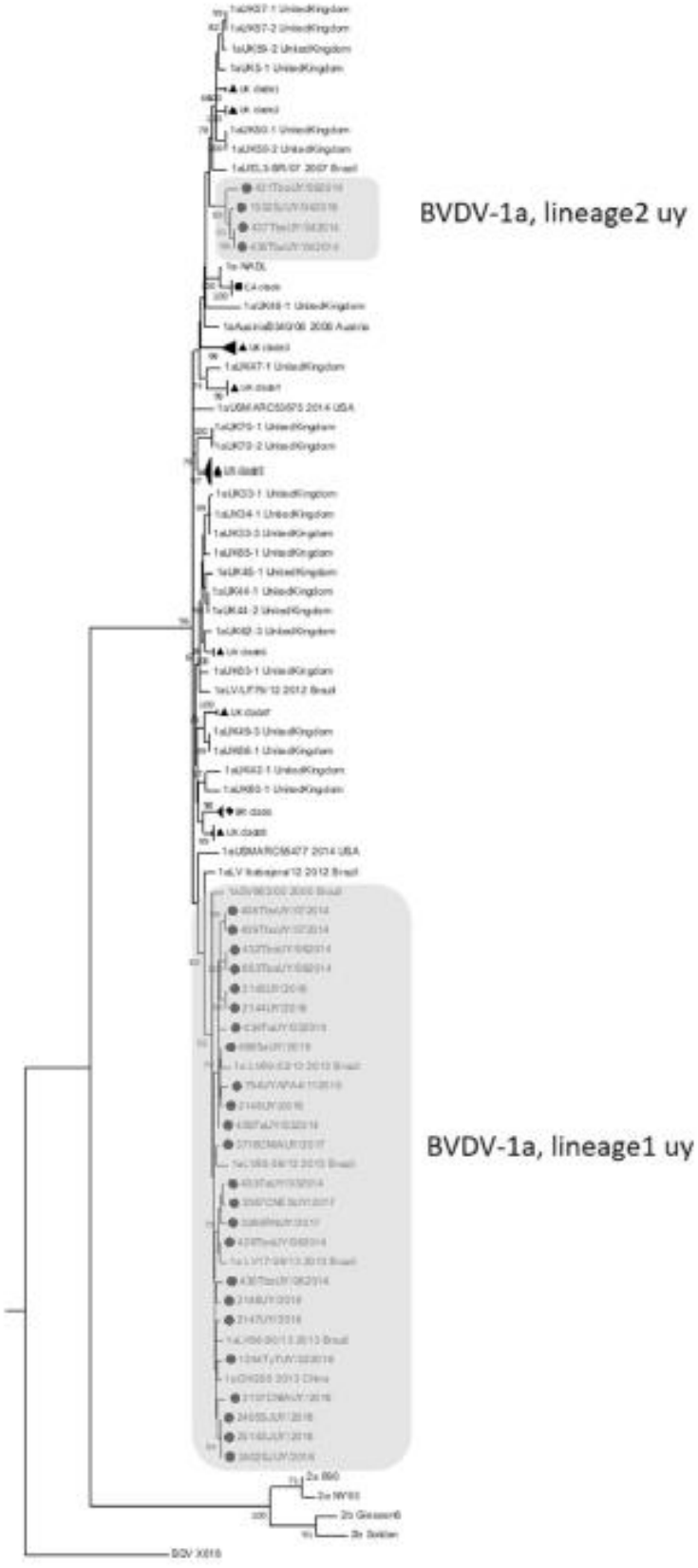

Fig. 2 Phylogenetic analysis of N^{pro} sequences of BVDV-1a strains. Uruguayan BVDV-1a strains are indicated by dots. The UK clade is indicated by triangles, the CA clade by squares, and the BR clade by rhombuses. A BVD (border disease virus) sequence was included in the analysis as an out-group

Table 4 BEAST results for the 5'UTR/N^{pro} genomic region of BVDV-1a Uruguayan strains

	Value	95% HDP
tMRCA	1990	1968-2007
Substitutions/site/year	3.48×10^{-3}	9.98×10^{-4}-5.96×10^{-3}

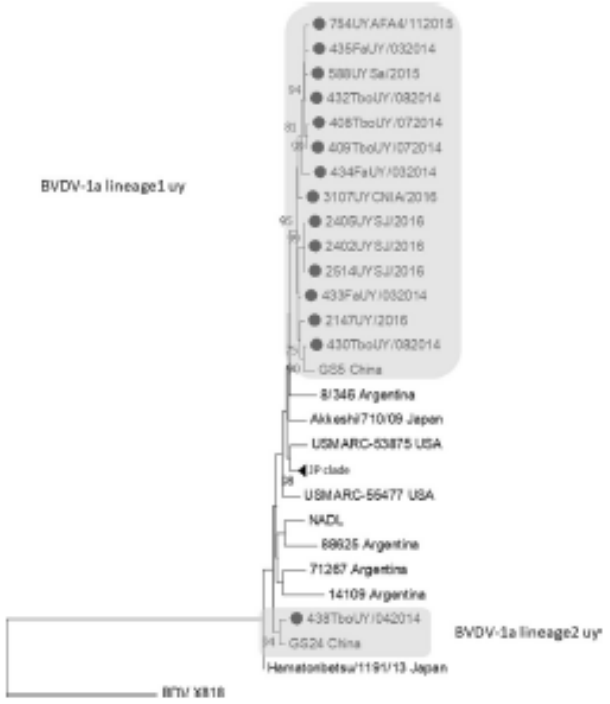

Fig. 3 Phylogenetic analysis of E2 nucleotide sequences of BVDV-1a strains. Uruguayan BVDV-1a strains are indicated by dots. A BDV (border disease virus) sequence was included in the analysis as an out-group

group", with 98% statistical support. Strains from Argentina did not cluster together, revealing a significant degree of genetic variability among them.

Discussion

BVD is one of the most important infectious diseases of livestock, causing significant economic losses to the livestock industry. In Australia, the beef and dairy industries are among the top three in the agricultural sector. In Australia, there are no mitigation programs for BVD, and this disease is second in importance to diseases carried by ticks. In tick-free areas, BVDV is the most important pathogen [36].

In Uruguay, cattle industry exports represent around 10% of the country's gross domestic product [37]. There

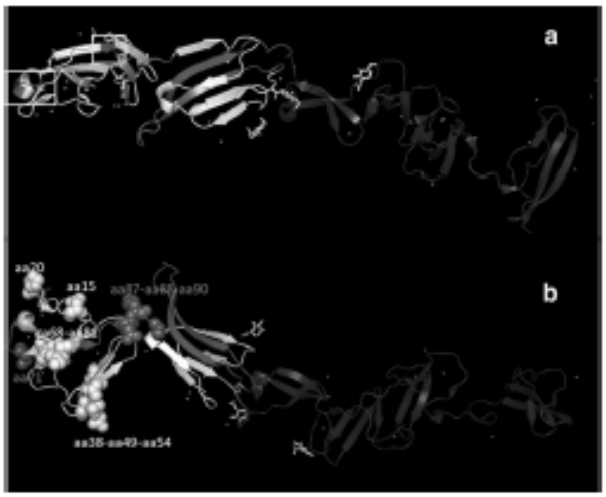

Fig. 4 Cartoon representation of the E2 monomer. **a)** The amplified region of the E2 glycoprotein is shown in white, and the rest of the monomer is in dark grey. Antigenic sites mapped in classical swine fever virus and BVDV are shown in light grey and indicated by squares. **b)** Cartoon representation of the E2 monomer rotated about the y-axis 180 degrees counterclockwise. The 11 amino acid substitutions found in the Uruguayan strains of the BVDV-1a subtype are represented as spheres colored light grey if located in a known antigenic site. Otherwise, they are shown in white

is a cattle population of around 12 million head, including beef and dairy breeds, distributed on 42,114 farms. The bovine population in Uruguay has remained practically unchanged since 1998, and it is suspected that BVDV is one of the factors that contribute to this situation, affecting pregnancy and procreation [38]. Research studies carried out in Uruguay in 2000 revealed that only 3% of farmers vaccinated cattle against BVDV. BVDV was found on 100% of the farms studied, and a seroprevalence of 67% was found in cattle [39]. These data were updated recently with similar values of seroprevalence at the farm level (98.8%) and higher seroprevalence at the herd level (approximately 80%, Dr. Federico Fernández, Ministerio de Ganadería, Agricultura y Pesca, MGAP, personal communication). Yarnall and Thrusfield estimated that farms endemically infected with BVDV could experience an economic impact ranging from 8.4 to 113 dollars per cow per year [4].

In Uruguay, there is no national program for the eradication of BVDV, and vaccination is not mandatory. The vaccines against BVDV available in Uruguay are listed in the national annual report to the World Organization for Animal Health (OIE); they are inactivated polyvalent vaccines that contain BVDV-1 and BVDV-2 [40]. Only one of the manufacturers states that the vaccine contains the NADL reference strain of the BVDV-1a subtype and the SV323 strain of the BVDV-2a subtype. No information

is available on the viral subtypes included in the vaccines prepared by other manufacturers.

Many countries, such as Austria, Great Britain, Denmark, Finland, France, Germany, Ireland, Italy, the Netherlands, Norway, Scotland, Slovenia, Sweden and Switzerland have BVDV eradication programs in progress at the national or regional level [36]. In Switzerland, the virus has been successfully eradicated [41].

BVDV eradication can lead to a significant reduction of economic losses. To achieve this goal, a control policy should be implemented consisting in identification and removal of PI animals, identification of natural *in utero* infection of calves, continued surveillance of animals showing clinical signs characteristic of BVD, and an effective vaccination program [42, 43].

In our previous work, we found that BVDV-1 and BVDV-2 were circulating in Uruguayan herds with reproductive problems and that the most frequent subtype was BVDV-1a [19]. The current work extends the sampling and supports the molecular epidemiology data obtained previously, given that the BVDV-1a subtype continues to be the most frequent subtype in our country.

The geographical diversification of the Uruguayan strains of BVDV-1a described in our previous work became more evident when increasing the number of positive samples of this subtype. Phylogenetic analysis clearly showed two clades of Uruguayan BVDV-1a (Figs. 1, 2 and 3). These clades were named "BVDV-1a lineage 1 UY" and "BVDV-1a lineage 2 UY". BVDV-1a lineage 1 UY included a larger number of Uruguayan strains (n = 24) and formed a clade together with some Brazilian strains and the GS5 strain isolated in China (Figs. 1 and 2). In contrast, BVDV-1a lineage 2 UY included fewer Uruguayan strains (n = 4) and only one foreign strain, GS24 of Chinese origin (Fig. 3).

According to the tMRCA estimate, the BVDV-1a subtype began circulating in Uruguay in 1990, approximately 27 years ago. Since then, this subtype began to spread and evolve, accumulating substitutions at a rate of 3.48×10^{-3} substitutions/site/year (s/s/y) in the 5'UTR/N^{pro} region. In this way, the BVDV-1a subtype in Uruguay diversified by acquiring local specific genetic characteristics that gave rise to two BVDV-1a lineages, which are genetically different from the BVDV-1a strains isolated in other countries and from the vaccine strain NADL. The estimates of the substitution rates found in these analyses (3.48×10^{-3} s/s/y) are consistent with those reported in previous studies [22, 44].

The results of these studies revealed that both of the BVDV-1a lineages circulating in Uruguay were genetically distant from the NADL vaccine strain (see Figs. 1-3). In the N^{pro} protease region, the two Uruguayan lineages differed from the NADL strain by four amino acid changes $N/E_{30}D$, $I_{39}V$, $K_{75}R$, $F_{110}S$ (Table 3a).

In order to obtain more-detailed information on the divergence between the NADL vaccine strain and the Uruguayan BVDV-1a strains, we analyzed the amino-terminal region of glycoprotein E2. This region is more variable than the N^{pro} region due to its role in the replicative cycle of the virus. The amino-terminal region of E2 is the most exposed region on the surface of the virus and therefore is the most antigenic region of this protein [20]. In the E2 region, most of the nucleotide changes are non-synonymous and lead to amino acid changes (see Table 2). In this region, 11 amino acid changes were found when comparing the two Uruguayan lineages of BVDV-1a and the NADL strain, revealing a significant degree of genetic variability among vaccine strain and strains circulating in Uruguay (Table 3b, Fig. 4). Substitutions at residues 71, 87, 88 and 90 found in the two BVDV-1a lineages circulating in Uruguay relative to the NADL strain are part of two epitopes of E2 [20, 35]. Therefore, these substitutions in glycoprotein E2 of the Uruguayan strains could lead to a protection/neutralization failure in animals vaccinated with the NADL strain. More studies will be needed to address this important issue.

In addition to the mutations described above, the Uruguayan sequences from BVDV-1a lineage 1 UY had a unique fingerprint $T_{38}V_{54}T_{68}E_{81}$, in glycoprotein E2, which differentiates them from strains of BVDV-1a lineage 2 UY ($M_{38}M_{54}I_{68}K_{81}$), the NADL strain ($K_{38}M_{54}I_{68}K_{81}$), and also the rest of the strains of the BVDV-1a subtype included in these analyses (Table 3b, shown in white, Fig. 4a and b). Although the amino acid changes at residues 38, 68 and 81 were not within previously reported antigenic sites, they might cause changes in the properties of the E2 protein. The mutation at residue 54 of BVDV-1a lineage 1 UY resulted in a smaller R group in the valine residue than in the methionine residue shared by the NADL vaccine strain and members of BVDV-1a lineage 2 UY. More studies are needed to determine whether these amino acid changes are relevant for the fitness of the Uruguayan strains with this amino acid fingerprint.

The greatest diversity of BVDV-1 subtypes has been described in Europe. The BVDV-1i subtype was first described in the United Kingdom in 1999. It appeared to be restricted to that country until 2014, when it was found in Uruguay, and in 2016, it was also found in Brazil [19, 45, 46]. The strains from the United Kingdom grouped together with 99% statistical support and were separated from the strains from Brazil and Uruguay (Fig. 1). Strains isolated in South America have a close genetic relationship with each other in the N^{pro} region and a more distant genetic distance with strains isolated in the United Kingdom [46].

In contrast to the BVDV-1, the BVDV-2 is not common in South America. However, BVDV-2b is the predominant subtype in Brazil [9]. The two Uruguayan strains belonging to the BVDV-2b subtype (2391UYRN/2016 and

2769UYRN/2016) clustered together, separated from most of the other strains of this subtype, including the Uruguayan strain 439RvUY/082014. More studies will be needed in order to observe if genetic diversification is taking place within the BVDV-2b genotype in Uruguay.

The relationship between the Brazilian, British, Chinese and Uruguayan strains (BVDV-1a, 1i and BVDV-2b) could probably be explained by the movement of the virus between these countries through commercial relationships in the livestock sector.

In summary, BVDV is a major health problem in Uruguayan herds, affecting production and reproduction. Appropriate prevention measures should be applied in order to control this pathogen and reduce the economic losses it causes. This study provides information about the molecular epidemiology of BVDV in our country. The BVDV-1a subtype is the most prevalent in Uruguay, and it has evolved over three decades, acquiring local characteristics that make it different from the NADL vaccine strain.

The Uruguayan subtype BVDV-2b should be included in the vaccine formulation, even though only a few isolates of this subtype were found. BVDV-2b also seems to be diverging in the Uruguayan territory, so surveillance of BVDV needs to be continued in order to have a clear picture of the behavior of this viral subtype in our country.

The results of these studies suggest that, to achieve adequate prophylaxis, it might be adequate to reformulate existing vaccines to contain the two types of BVDV and the subtypes BVDV-1a and BVDV-2b.

Additional in-depth studies are needed to investigate the antigenic characteristics and determine the complete genome sequences of Uruguayan BVDV strains in order to select local isolates as candidates for developing a suitable protective vaccine for our herds.

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Capítulo 4_ Manuscrito 4:

Macías-Rioseco, Melissa, Silveira, Caroline, Fraga, Martin, Casaux, Laura, Cabrera, Andrés, Francia, María E., Robello, Carlos, **Maya, Leticia**, Zarantonelli, Leticia, Suanes, Alejandra, Colina, Rodney, Buschiazzo, Alejandro, Giannitti, Federico, & Riet-Correa, Franklin. (2020). Causes of abortion in dairy cows in Uruguay. *Pesquisa Veterinária Brasileira*, *40*(5), 325-332. Epub July 17, 2020. https://doi.org/10.1590/1678-5150-pvb-6550

En este trabajo se analizaron 102 abortos bovinos para determinar las causas de aborto y las frecuencias de las mismas. Todos los casos se sometieron a exámenes patológicos macroscópicos y microscópicos, así como a pruebas microbiológicas y serológicas. La etiología se determinó en 54 (53%) de los casos, 51 de los cuales fueron causados por agentes infecciosos.

Los principales hallazgos de este trabajo en relación a BVDV se resumen a continuación:

- En 3 fetos se detectó a BVDV
- 2 de estos fetos estaban coinfectados con *Neospora caninum*
- En el tercer feto positivo a BVDV, se detectó la coinfección con *Leptospira interrogans*

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Original Article
Livestock Diseases

Causes of abortion in dairy cows in Uruguay

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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 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 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: Dairy cattle, Uruguay, bovine abortion, *Campylobacter fetus*, *Coxiella burnetii*, *Neospora caninum*, cattle.

RESUMO.- **[Causas de aborto em bovinos de leite no Uruguai.]** Uma série de casos foi estudada para determinar a frequência de causas do aborto em bovinos leiteiros no Uruguai. A amostra, de 102 casos, foi composta por 53 fetos, 35 fetos com placentas e 14 placentas sem feto associado. Todos os casos foram submetidos a exames patológicos macroscópicos e microscópicos, além de testes microbiológicos e sorológicos. A etiologia foi determinada em 54 (53%) dos casos, 51 dos

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quais foram causados por agentes infecciosos. Nos 102 casos observados, 30 (29%) foram causados por *Neospora caninum*, seis (6%) por *Coxiella burnetii* e dois (2%) por *Campylobacter fetus* subsp. *venerealis*. O vírus da Parainfluenza-3 e *Salmonella enterica* serovar Newport causaram um aborto cada. Bactérias oportunistas (*Escherichia coli, Streptococcus* sp., *Staphylococcus* sp., *Mannheimia* sp., *Trueperella pyogenes* e *Providencia stuartii*) foram associadas a 11 abortos. Em dois casos, a morte fetal foi atribuída a distocia e, em um caso, o feto apresentava mesotelioma congênito. A infecção pelo vírus da diarreia viral bovina (BVDV) foi identificada em três fetos; dois dos quais foram co-infectados e apresentavam lesões típicas de *N. caninum*. Não foram observadas lesões no outro feto infectado pelo BVDV. *Leptospira interrogans* foi identificada em um feto sem lesões. Apesar da relativamente baixa taxa de sucesso no diagnóstico etiológico nos casos de aborto em bovinos, é necessário o diagnóstico sistemático dos abortos para estabelecer estratégias de prevenção e controle. Isso também facilita o monitoramento e a vigilância de doenças reprodutivas em bovinos leiteiros, algumas das quais representam um risco para a saúde pública.

TERMOS DE INDEXAÇÃO: Aborto bovino, bovinos de leite, Uruguai, *Campylobacter fetus*, *Coxiella burnetii*, *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 a total of 494 bovine fetuses from 270 farms. 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 lesions 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. 2019a). 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.

Pathologic 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, forestomachs, 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-3 virus, and/or *N. caninum* were performed in selected tissues in some cases (see below). At necropsies, samples were collected for bacteriology, molecular biology, and serologic 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 where 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/IA, 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 RT-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 et al. (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. Heat induced antigen retrieval was performed 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-3 (BPI-3) virus 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. 2019b).

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 IHC procedures for *Chlamydia* spp. and *C. burnetii* and the PCR in FFPE placenta in these same cases has recently been published by our group (Macías-Rioseco et al. 2019c).

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 et al. (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 BPI-3 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 the fetus and placenta were received 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).

The 30 abortions caused by *N. caninum* (55.6% of the 54 cases with determined etiology) were from different dairy farms, accounting for 53.3% of the 45 farms included in the study. Of the six 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). 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, and BPI-3 virus 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. 2019a). 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 (29 fetuses), myocarditis (24), myositis (diaphragm or tongue) (20) hepatitis (15), interstitial nephritis (10) and/or interstitial pneumonia (6). Additionally, placenta was submitted along with the fetus in 10 of these 30 cases, 7 of which had placentitis. 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 *C. 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

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

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. BPI-3 virus 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. 2019b). In cases with lesions but without an identified etiology, the lesions consisted of myocarditis, myositis, glossitis, cerebral gliosis, nephritis, pneumonia, and hepatitis.

Brucella abortus was not 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 this agent. *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 BVDV-positive 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. 2019c). We were able to identify abortions due to coxiellosis based on the microscopic

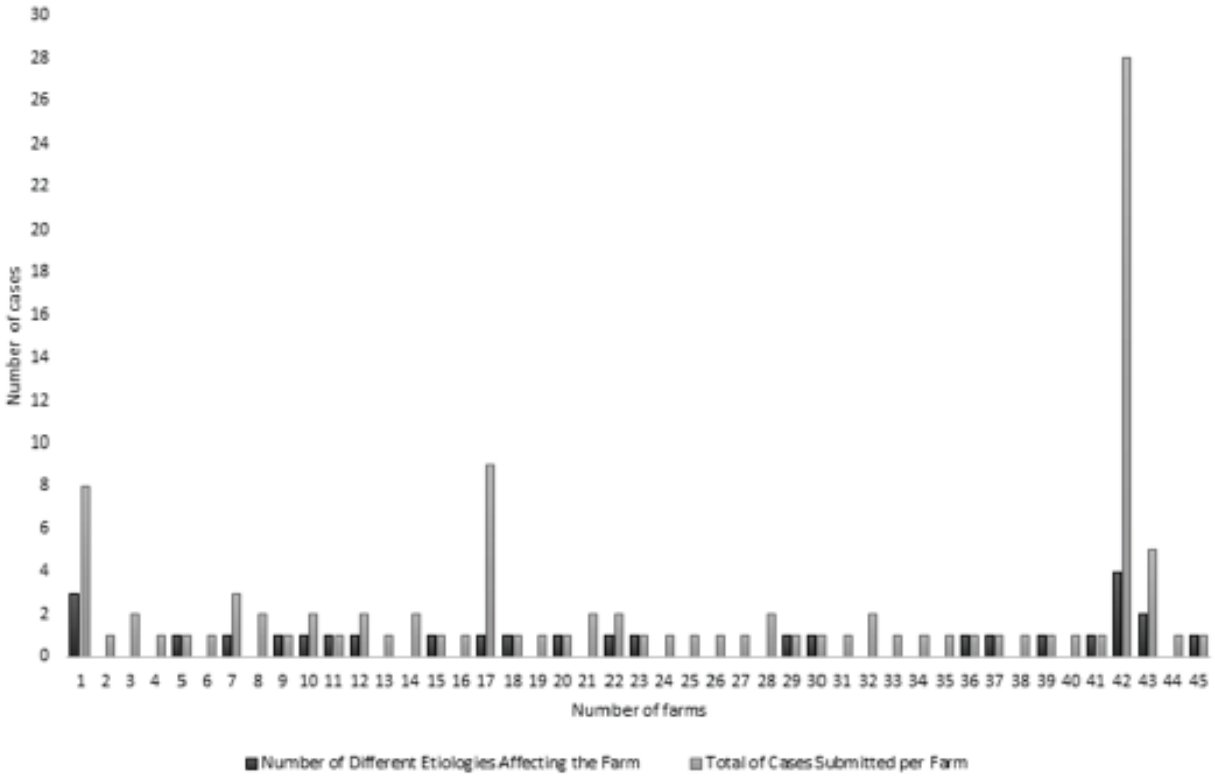

Fig.1. Frequency of different etiologies affecting each farm and total number of cases submitted per farm.

evaluation of the placenta followed by IHC along with PCR for *C. burnetii*. In all cases, the diagnosis of coxiellosis 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 coxiellosis (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 venereal 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 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 leptospires 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 further 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 (Vaucher et al. 2011, Clothier & Anderson 2016). In this case series, one abortion was caused by BPI-3 virus. 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. 2019b). 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), and 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, and most likely infertility in dairy cattle in the country.

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.

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Este trabajo tuvo como cometido evaluar en terneros de tambos patógenos asociados al complejo de Diarreas neonatales de terneros y las muertes, y si estas infecciones, diarreas o muertes se asociaban a la falla de transferencia de inmunidad pasiva.

Para BVDV se evaluaron suero o sangre de 231 terneros diarreicos y 249 terneros no diarreicos, de 1 a 30 días de edad provenientes de 27 establecimientos.

Los principales hallazgos de este trabajo en relación a BVDV se resumen a continuación:

- 6 animales pertenecientes a 4 establecimientos fueron positivos a BVDV
- 4 de los animales positivos tenían diarrea, y 2 animales no tenían diarrea
- De acuerdo con los resultados de este trabajo no se encontró una asociación entre el complejo de las diarreas neonatales y la infección con BVDV. Se presume que esta falta de asociación se podría deber al bajo número de casos positivos a BVDV.

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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Causes of neonatal calf diarrhea and mortality in pasture-based dairy herds in Uruguay: a farm-matched case-control study

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Abstract

Neonatal calf diarrhea (NCD) and mortality cause significant losses to the dairy industry. The preweaning dairy calf mortality risk in Uruguay is high (15.2%); however, causes for these losses are largely unknown. This study aimed to assess whether various pathogens were associated with NCD and death in Uruguayan dairy calves and whether these infections, diarrhea, or deaths were associated with the failure of transfer of passive immunity (FTPI). Contemporary diarrheic ($n = 264$,) and non-diarrheic ($n = 271$) 1- to 30-day-old calves from 27 farms were sampled. Feces were analyzed by antigen-capture ELISA for *Cryptosporidium* spp., rotavirus, bovine coronavirus, and *Escherichia coli* F5+, RT-PCR for bovine astrovirus (BoAstV), and bacterial cultures for *Salmonella enterica*. Blood/serum was analyzed by RT-PCR or antigen-capture ELISA for bovine viral diarrhea virus (BVDV). Serum of ≤ 8-day-old calves ($n = 95$) was assessed by refractometry to determine the concention of serum total proteins (STP) as an indicator of FTPI. Whether the sampled calves died before weaning was recorded. At least one pathogen was detected in 65.4% of the calves, and this percentage was significantly higher in diarrheic (83.7%) versus non-diarrheic (47.6%) calves. Unlike the other pathogens, *Cryptosporidium* spp. and rotavirus were associated with NCD. Diarrheic calves, calves infected with any of the pathogens, and calves infected with rotavirus had significantly lower concentrations of STP. Diarrheic calves had higher chances of dying before weaning than non-diarrheic calves. Diarrheic calves infected with *S. enterica* were at increased risk of mortality. Controlling NCD, salmonellosis, cryptosporidiosis, and rotavirus infections, and improving colostrum management practices would help to reduce calf morbi-mortality in dairy farms in Uruguay.

Keywords *Cryptosporidium* spp. · Dairy calves · Diarrhea · Failure of transfer of passive immunity · Infectious diseases · Mortality · Rotavirus · *Salmonella enterica*

Introduction

Dairy farming in Uruguay is socio-culturally and economically important, as Uruguay is one of the top per capita consumers of dairy products in Latin America [1], and approximately 70% of the milk produced in the country is exported [2]. Uruguayan dairy farming systems are largely pasture-based, with 75% of the diet of the milking herd being

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farm-grown forage [3]. From 1985 to 2016, milk production grew linearly at a rate of 3.2% per year, with an attendant reduction in the number of farmers and the area allocated to dairy farming. This growth was due to increased stocking rates, individual milk production and the milking cow:dry cow ratio [2], indicating a production intensification process. However, the national dairy stock did not grow significantly over the past decade [2], which has partly been attributed to a relatively high (15.2%) nationwide annual dairy calf mortality risk from birth to weaning [4]. Despite this, little information is available in the scientific literature on the causes of dairy calf diseases and death in this country.

Neonatal calf diarrhea (NCD) is the leading cause of dairy calf morbidity and mortality within the first month of life [4, 5]. Affected calves suffer from dehydration, electrolyte imbalances, and metabolic acidosis, which, if left untreated, can lead to death [6]. Long-term effects of NCD in dairy heifers include reduced weight gain and development, increased time to first calving and reduced milk production in the first lactation, which result in significant economic losses to the livestock sector [7, 8]. As the leading cause of dairy calf morbidity and mortality, NCD also raises serious concerns on newborn calf welfare [9] and the excesive use of antibiotics with potential increase of antibiotic resistance [10, 11].

NCD is a complex and multifactorial syndrome, it can be caused by several infectious and parasitic agents, including viruses (e.g., rotavirus, bovine coronavirus -BCoV-, and bovine viral diarrhea virus -BVDV-), bacteria (e.g., enterotoxigenic and enteropathogenic/enterohemorrhagic *Escherichia coli* and *Salmonella enterica*), and protozoa (e.g., *Cryptosporidium* spp. and *Eimeria* spp.) [12–15], some of which are zoonotic. Although under field conditions BVDV rarely causes diarrhea in neonatal calves that receive colostral antibodies, it can be associated with perinatal and neonatal mortality due to congenital infections [16, 17]. Other agents that infect neonatal calves, such as bovine astrovirus (BoAstV), have been suspected to play a causative role in NCD, although attempts to experimentally reproduce diarrhea in gnotobiotic calves have been unsuccessful [18], and information available from field studies is limited [15]. Studying causality in spontaneous outbreaks of diseases with multifactorial etiologies is challenging and requires extensive laboratory testing for various pathogens. Agents that cause NCD can be found both in diarrheic and non-diarrheic calves, and the same or different calves in an outbreak can be coinfected by two or more causative agents [19–21], making interpretation of individual test results difficult in clinical contexts.

Additionally, NCD is frequently associated with nutritional and/or immunological factors, such as failure in the transfer of passive immunity (FTPI) [8, 22], and environmental and management factors that either favor the transmission of the causative agents [20] or increase the susceptibility of the calves. Transfer of passive immunity is arguably the single most important non-infectious factor determining neonatal calf health and survival [23]. Calves with FTPI are at increased risk of disease and mortality [22, 24], and a large proportion of calf deaths up to 3 weeks of life can be attributed to FTPI [5, 25].

NCD outbreak investigations should be comprehensive and consider not only the infectious and parasitic etiological agents in affected and unaffected calves, but also epidemiological aspects and herd management practices that can vary greatly between farms [26]. In this context, farm-matched case-control studies represent adequate designs for the evaluation of possible associations between single or mixed infection and clinical outcomes, to better understand the causal role of the different agents while minimizing confounders. NCD has been broadly studied through cross-sectional [19, 20, 27, 28] and, to a lesser extent, case-control designs [21, 29, 30]; although these case-control studies were not matched considering the farms of origin of the calves, somewhat limiting the conclusions that can be drawn from them.

Because causes of NCD and mortality in Uruguayan dairies are largely unknown, in this study, we aimed at assessing the association of several known (*Cryptosporidium* spp., rotavirus, BCoV, *E. coli* F5+, *S. enterica*), putative (BoAstV) and occasional (BVDV) pathogens for calves with diarrhea and/or death, and whether these infections, diarrhea, or death were associated with FTPI through a farm-matched case-control study.

Materials and methods

Study design

A case-control study was conducted in 27 pasture-based, commercial dairy farms in six Uruguayan departments (San José, Río Negro, Colonia, Flores, Florida and Soriano) between January and November 2016. Farms were sampled by convenience because they were experiencing spontaneous outbreaks of NCD. In all farms, Holstein was either the only or the predominant breed of cattle; two farms had some Holstein-Jersey crosses. Contemporary calves with diarrhea (cases) and a similar number of non-diarrheic calves (controls) were sampled in each farm. Only calves aged 1–30 days were included; control calves were within an age range of 0–13 days of their respective cases. On average, 19.8 calves per farm (range: 13–29) were sampled, totaling 535 calves. Overall, 49.3% (n = 264) of the calves included in the study were experiencing diarrhea (cases) at the time of sampling, and the remainder 50.7% (n = 271) were not experiencing diarrhea and did not have a history of diarrhea (controls). The overall case:control ratio was 1:1.03. The sample size was calculated using an online epidemiological calculator [31], considering a power of 80% to detect an association between diarrhea and a

given infectious/parasitic agent, a percentage of exposed controls of 5%, and an Odds Ratio (OR) of 2.5 with a 95% confidence level.

Animal specimens

Individual fecal, whole blood, and serum samples were obtained from each calf by a veterinarian, following procedures approved by INIA´s animal ethics committee for the use of animals in experimentation (protocol #20199). Fecal samples were collected from the rectum using individual sterile fecal cups and gloves. A fecal score was assigned to each sample as previously described [32], based on which calves were classified as either non-diarrheic (controls; fecal score ≤ 1) or diarrheic (cases; fecal score ≥ 2). Additionally, the fecal samples were assessed macroscopically for the presence of fibrin and/or mucus. Blood samples were drawn by jugular venipuncture using individual sterile needles and syringes and collected in red top tubes for serum and heparinized tubes (BD Vacutainer, Franklin, NJ) for whole blood.

Data collection

At the end of the calf-rearing period in each of the 27 farms, a questionnaire was conducted to the farmers to assess whether the individual calves sampled for this study had died within the rearing period (before weaning), until 60 days of age (follow-up time), and the age in days at death, when available (Supplementary Material 1).

Pathogen detection in feces

Enzyme-linked immunosorbent assay (ELISA) for coproantigen detection

A commercial monoclonal antibody-based antigen-capture ELISA kit (Pathasure Enteritis 4, Biovet Inc., St-Hyacinthe, Canada) was used to detect *Cryptosporidium* spp., rotavirus, BCoV and *E. coli* F5+ (K99+) antigens in fresh feces from all 535 calves [33], 24–72 h after sample collection, following the manufacturer´s recommendations.

Salmonella enterica culture and serotyping

All 535 fecal samples were cultured aerobically in tetrathionate broth (Oxoid, code CM0671) for 24–48 h at 37°C (selective enrichment) after which 100 μl of broth were plated onto xylose-lysine-deoxycholate (XLD) agar (Oxoid, code CM0469). Suspect colonies were selected, and routine biochemical tests were performed for identification of *S. enterica*, as previously described [34]. *Salmonella enterica* serotyping was performed following the Kauffman-White-Le Minor classification scheme [35] at the bacteriology service of the "Instituto de Higiene, Facultad de Medicina, Universidad de la República," in Montevideo, Uruguay.

RT-PCR for bovine astrovirus

A total of 396 fecal samples were diluted 1:10 (v:v) in phosphate-buffered saline solution and centrifuged at 3000*g* at 4°C for 20 min. Supernatants were collected and stored in a freezer at −80°C. Viral RNA was extracted using QIAamp cador Pathogen Mini Kit (Qiagen) with an elution volume of 50 μL. Reverse transcription (RT) was carried out with RevertAid Reverse Transcriptase (ThermoFisher, Scientific) and random hexamer primers (Qiagen) to obtain cDNA that was stored at -20°C. BoAstV PCR was performed using MangoMix (Bioline) and primers BoAstV-F and BoAstV-R that amplify a 432-nucleotide fragment of the polymerase gene of BoAstV, as described elsewhere [36]. PCR products were visualized in 2% agarose gels. The results were expressed as positive or negative.

Bovine viral diarrhea virus detection in blood or serum

Frozen samples of serum ($n = 418$) or whole blood ($n = 62$) were processed for BVDV detection, either by a commercial antigen-capture ELISA (BVDV Ag/Serum Plus Test, IDEXX, Switzerland) (136 serum and 62 whole blood samples) or by RT-PCR (282 serum samples). The RT-PCR was performed as previously described [37].

Assessment of transfer of passive immunity in neonatal calves

Serum samples from all calves that were ≤ 8 days of age at the time of sampling ($n = 95$) were analyzed for serum total solids using an optic refractometer (ATAGO PAL-1, Tokyo, Japan), as an indicator of the concentration of serum total proteins (STP) in g/dl. A cutoff value of < 5.6 g/dl was considered to determine FTPI, as previously described [38]. Additionally, the results were categorized to reflect calves with poor (< 5.1 g/dl), fair (5.1–5.7 g/dl), good (5.8–6.1 g/dl), or excellent (> 6.2 g/dl) transfer of passive immunity as suggested by Godden et al. [23].

Data and statistical analyses

Data of each sampled calf, including the age at sampling, farm of origin, occurrence of diarrhea, presence or absence of fibrin and/or mucus in feces, all laboratory test results (pathogen detection, STP concentration), and the information from the questionnaire, including whether calves had died or survived during the preweaning period (follow-up time) was collected. Data was entered into a Microsoft Excel 2013 spreadsheet to

create a digital database (Supplementary Material 1) that was used as a template for statistical analyses. A brief description of the statistical analyses are presented in the following paragraphs; a more detailed description is available in Supplementary Material 2.

Descriptive statistics of the raw data including proportions, means, and standard deviations (SD, for data with normal distribution), median and interquartile range (IQR, for data not normally distributed) were calculated. Differences between the proportions of diarrheic calves by age in weeks, the proportions of diarrheic and non-diarrheic calves that tested positive to at least one of the pathogens and to ≥ 2 pathogens, and the proportions of diarrheic and non-diarrheic calves that died before weaning were assessed by chi-square. Differences in the STP concentrations (g/dl) in diarrheic versus non-diarrheic calves, as well as calves that tested positive or negative for (a) given pathogen(s) were evaluated by analyses of variance using the procedure PROC MIXED (SAS University Edition, SAS Institute Inc. Cary, NC, USA), controlling for herd as a random effect. Results were expressed in least squares means (LMS) and standard errors of the means (SEM).

A logistic regression model was fit to assess the effect of the pathogens on diarrhea. The model accounted for the structure of the sample design in which diarrheic calves (cases) were matched with control calves within a farm [39]. The variable "age" grouped in weeks (1, 2, 3, and > 3) was included in the model to adjust the OR. Interaction terms between the pathogens were tested to assess the effects of co-infections. The model was made using the procedure PROC LOGISTIC including the farm identification in the STRATA statement; the model fit was checked by the Akaike Information Criterion (AIC) and *r* squared [22, 40, 41].

A univariate generalized estimating equation (GEE) repeated-measures logistic model was made to assess the effect of the concentration of STP on diarrhea, rotavirus, BCoV, and *E. coli* F5+ detection adjusted by the age of the calves in days. This hypothesis was tested because vaccination against these pathogens is a common practice in Uruguayan dairy farms. For this model, values of STP concentration < 5.6 g/dl were classified as low and compatible with FTPI, as previously described [38].

A multivariate GEE repeated-measures logistic model was employed to assess the association between the presence of fibrin or mucus in the feces and the pathogens adjusted by age of the calves in weeks.

Finally, we tested the risk of death among diarrheic calves infected with *S. enterica*, rotavirus and *Cryptosporidium* spp. adjusted by age in days using the same multivariate GEE model structure but including the Poisson instead of the binomial distribution to estimate the risk of death [42]. BCoV, BoAstV, BVDV, and *E. coli* F5+ were not included because there were either only one (BCoV, BVDV and *E. coli* F5+) or too few (BoAstV) deceased diarrheic calves infected with these pathogens. For this, data from all the diarrheic calves for which the questionnaire was available (118 calves) was used; these calves were followed-up until the end of the pre-weaning period.

A significance level (alpha) of 5% ($p < 0.05$) was considered for all the statistical analyses.

Results

At the time of sampling, the calves had a median of 11 days of life (IQR = 9), with a range of 1 to 30 days [non-diarrheic calves = 12 (IQR = 14), diarrheic calves = 11 (IQR = 5)]. The proportion of diarrheic calves was 39.9% (61/153) in the first week of life, 71.8% (158/220) in the second, 28.9% (33/114) in the third and 25.0% (12/48) after the third week of life. The proportion of diarrheic calves was significantly higher in the second week of life ($p < 0.001$). Fibrin or mucus were observed in the feces of 8.1% (21/259) and 47.1% (122/259) of diarrheic calves and 2.6% (7/266) and 32.3% (86/266) of non-diarrheic calves, respectively.

The questionnaire was completed for 241 calves (118 cases and 123 controls), 19.9% (48/241) of which had died before weaning at 5–37 days of age (median age at death: 16, IQR = 13). The remainder 80.1% (193/241) of the calves survived after weaning (> 60 days of age). Thirty-one (64.6%) of the calves that died before weaning were diarrheic, and the remainder 35.4% (17/48) were non-diarrheic at the time of sampling. Of the 193 calves that survived after weaning, 45.0% (87/193) were diarrheic, and the remainder 55.0% (106/193) were non-diarrheic at the time of sampling. The proportion of calves that died before weaning was significantly higher among diarrheic than non-diarrheic calves ($p < 0.02$). Individual results for each calf in each farm are shown in Supplementary Material 1.

Detection of pathogens in feces and BVDV in serum/blood

At least one of the pathogens was detected in 65.4% (350/535) of the calves, this percentage was significantly higher in diarrheic (83.7%, 221/264) versus non-diarrheic (47.6%, 129/271) calves ($p < 0.001$). In 34.6% (185/535) of the calves no agents were detected, being 76.8% (142/185) of them non-diarrheic and the remaining 23.2% (43/185) diarrheic ones. Individual pathogens and coinfections were identified in 43.2% (231/535) and 22.2% (119/535) of the calves, respectively. The frequencies of detection of each pathogen individually and in coinfections are shown in Table 1and Supplementary Material 1. *Cryptosporidium* spp., BoAstV and rotavirus were the most frequently detected agents, both at the animal and farm levels (Table 1). *Cryptosporidium* spp. and rotavirus were detected with a significantly higher

Table 1 Overall frequency of detection of pathogens in 535 diarrheic and non-diarrheic dairy calves from 27 farms in Uruguay

Tests (sample type and total No. analyzed)	No. of samples analyzed from diarrheic and non-diarrheic calves	Total no. (%) of positive calves	Total no. (%) of diarrheic positive calves	Total no. (%) of non-diarrheic positive calves	No. of farms with ≥ 1 positive animal (% of total No. of farms, $n = 27$)
Cryptosporidium spp. ELISA (feces, 535)	264 and 271	256 (47.8%)	183 (69.3%)	73 (26.9%)	27 (100.0%)
BoAstV RT-PCR (feces, 396)	186 and 210	86 (21.7%)	39 (21.0%)	47 (22.4%)	21 (77.8%)
Rotavirus ELISA (feces, 535)	264 and 271	94 (17.6%)	64 (24.2%)	30 (11.1%)	24 (88.9%)
Salmonella enterica isolation (feces, 535)	264 and 271	21 (3.9%)	14 (5.3%)	7 (2.6%)	8 (29.6%)
Escherichia coli F5+ ELISA (feces, 535)	264 and 271	11 (2.1%)	6 (2.3%)	5 (1.8%)	5 (18.5%)
BCoV ELISA (feces, 535)	264 and 271	8 (1.5%)	5 (1.9%)	3 (1.1%)	5 (18.5%)
BVDV antigen ELISA or RT-PCR (serum/blood, 480)	231 and 249	6 (1.3%)	4 (1.7%)	2 (0.8%)	4 (14.8%)

ELISA enzyme-linked immunosorbent assay, *BoAstV* bovine astrovirus, *RT-PCR* reverse transcriptase polymerase chain reaction, *BCoV* bovine coronavirus, *BVDV* bovine viral diarrhea virus

frequency in diarrheic calves, in contrast to BoAstV, which as a single infection had a significantly higher frequency in non-diarrheic calves. The proportion of diarrheic calves infected with one pathogen was 59.3% (137/231). In the 119 calves with coinfections (84 cases and 35 controls), two (89.1%; 106/119) or three (10.9%; 13/119) pathogens were identified. A proportion of 69.8% (74/106) of the calves coinfected with two pathogens were diarrheic, while the remainder 30.2% (32/106) were non-diarrheic. Of the 13 calves coinfected with three pathogens, 76.9% (10/13) were diarrheic. The proportion of diarrheic calves coinfected with ≥ 2 pathogens was 70.6% (84/119). All isolated *Salmonella* strains were *S. enterica* subspecies *enterica* serotype Typhimurium, except for two isolates from one farm that were serotype Anatum.

Association between pathogens, diarrhea, and preweaning death

In the final multivariate conditional logistic regression model individual infections with *Cryptosporidium* spp. (OR = 1.65, 95% CI = 1.25–2.17, $p = 0.0004$), rotavirus (OR = 1.79, 95% CI = 1.36–2.35, $p < 0.0001$), and their interaction ($p = 0.0274$) were associated with diarrhea. No association was observed between diarrhea and infection with BCoV, *E. coli* F5+ or *S. enterica*. There was an interaction effect between *Cryptosporidium* spp. and rotavirus, which means that the effects of one of this pathogens on diarrhea differered according with the infection status by the other and viceversa. The odds of diarrhea were higher in calves infected with *Cryptosporidium* spp. that were negative for rotavirus (OR = 5.77, 95% CI = 3.47–9.61), and in calves infected with rotavirus that were negative for *Cryptosporidium* spp. (OR = 4.93, 95% CI = 2.31–10.54), than in calves that were positive for both agents. The model with interaction terms had the best fit compared with the full model and with the model without interaction according to the AIC (507.6) and r squared values (0.35). Calves in the second week of life had significantly higher odds of being diarrheic (OR = 2.16, 95% CI = 1.25–3.69, $p < 0.0001$), while the odds decreased in calves in the third week of life (OR = 0.6, 95% CI = 0.33–1.11, $p < 0.03$). There were no associations between diarrhea and infection with BVDV (OR = 0.55, 95% CI = 0.08–3.70, $p = 0.54$) or BoAstV (OR = 0.85, 95% CI = 0.48–1.50, $p = 0.58$).

Calves without diarrhea at the time of sampling had lower chances of dying before weaning (OR = 0.40, 95% CI = 0.19–0.84, $p < 0.02$) than diarrheic calves.

Pathogens and the presence of fibrin or mucus in feces

Individual infections with *S. enterica* and rotavirus were significantly associated with the presence of fibrin in the feces in the multivariate repeated-measures logistic model. The odds of *S. enterica*-positive calves presenting fibrin in the feces was 5.8 times greater than for *S. enterica*-negative calves (OR = 6.8, 95% CI: 2.4–18.9, $p < 0.001$), while the odds of rotavirus-positive calves presenting fibrin in the feces was 1.2 times greater than for rotavirus-negative calves (OR = 2.2, 95% CI: 1.1–4.4, $p = 0.03$). None of the evaluated pathogens were associated with the presence of mucus in the feces.

Transfer of passive immunity, diarrhea, pathogens, and preweaning death

The STP concentration was assessed in 95 ≤ 8-day-old calves with (n = 40) and without (n = 55) diarrhea, 45 of them (47.4%) had FTPI based on the cutoff proposed by [38] (STP < 5.6 g/dl). According to the four-level categorization suggested by Godden et al. [23], 28.4% (27/95), 26.3% (25/95), 13.7% (13/95) and 31.6% (30/95) of the calves had poor, fair, good, or excellent transfer of passive immunity, respectively. The overall mean STP concentration was 5.64 g/dl (SD = 0.9, min = 4, max = 8.6), the LSM for the STP concentration was 5.81 g/dl (n = 55, SEM = 0.20) in non-diarrheic calves, and 5.36 g/dl (n = 40, SEM = 0.19) in diarrheic calves, these differences were statistically significant (p = 0.0445). Similarly, the STP concentration was significantly lower in calves infected with any of the pathogens under study (n = 62, LSM = 5.40 g/dl, SEM = 0.16) than in those that were negative to all tested pathogens (n = 33, LSM = 6.08 g/dl, SEM = 0.21) (p = 0.0016). Although calves with concentrations of STP ≥ 5.6 g/dl had 54.0% lower odds of presenting diarrhea than calves with STP concentrations < 5.6 g/dl (OR = 0.46, 95% CI = 0.21–1.03, p = 0.059), this difference was not statistically significant.

The calves that tested positive only for rotavirus had significantly lower concentrations of STP (n = 13, LSM = 5.20 g/dl, SEM = 0.24) than the negative ones (n = 82, LSM = 5.67 g/dl, SEM = 0.16) (p = 0.0469). Calves with STP concentrations ≥ 5.6 g/dl had 64.0% lower odds of being positive to rotavirus than calves with concentrations of STP < 5.6 g/dl (OR = 0.36, 95% CI = 0.12–1.07, p < 0.07), this difference was not statistically signicant. There were no associations between the STP concentration and infections by BCoV or *E. coli* F5+, or with preweaning death.

Pathogens and risk of death during the preweaning period in diarrheic calves

The risk of death before weaning was 0.9 times higher in diarrheic *S. enterica*-positive calves than in diarrheic *S. enterica*-negative ones (RR = 1.90, 95% CI = 1.13–3.16, p = 0.015). The other evaluated pathogens were not associated with increased risk of death of diarrheic calves before weaning.

Discussion

NCD is one of the leading causes of death in dairy calves [5]. In coherence with this, in our study, calves that manifested diarrhea early in their lives had significantly higher chances of dying before weaning than contemporary non-diarrheic ones, indicating that controlling and preventing NCD regardless of its cause would aid in reducing calf mortality in Uruguayan dairy farms. As a multifactorial syndrome associated with various infectious and parasitic agents, as well as non-infectious factors [13], determining its etiology is a complex process that usually requires laboratory testing along with clinical and epidemiological investigations. In this work, the frequency of infection with various known or putative pathogens, and their association with NCD, the presence of fibrin or mucus in feces, FTPI and death, was evaluated through a large-scale farm-matched case-control study in contemporary calves with and without diarrhea. Case-control studies are key to identify the possible associations and the role of the agents in disease, since most of the causative agents of NCD can be found in clinically healthy calves, and therefore their mere presence does not warrant disease causality. Additionally, because NCD is a multifactorial syndrome, the interaction between different potential etiologies needs to be considered in studies aiming at assessing causality. Case-control studies with large numbers of dairy calves that investigate multiple possible etiologies of NCD and conduct statistical analyses to assess interactions between multiple agents to obtain robust and reliable epidemiological information are scant in the scientific literature [21, 29, 30].

In our study, at least one of the evaluated pathogens was detected in 65.4% of the 535 calves, and 83.7% of the 264 diarrheic calves. The attributable factor in exposed calves was 0.82 (not shown), meaning that 82% of the NCD cases may be attributed to the studied agents. This also suggests that other infectious (i.e., attaching and effacing -enteropathogenic and enterohemorrhagic- *E. coli*) or non-infectious (i.e., nutritional) factors not assessed in this study, may also be contributing to NCD in a smaller proportion of cases. In our study, the proportion of diarrheic calves was higher in the second week of life, which indicates that calves are exposed to diarrheagenic agents early after birth, as observed by other authors [20, 43]. A decline in the levels of colostral neutralizing antibodies in the intestine is the main determinant for the occurrence of infectious diarrhea during the second week of life [44, 45].

The most frequently detected agents were *Cryptosporidium* spp., BoAstV and rotavirus, both as individual infections and coinfections. This is remarkable given the high frequency of detection of these agents at the farm level. The detection frequencies of rotavirus in diarrheic (24.2%) or non-diarrheic (11.1%) calves in our study were much lower than those reported by RT-qPCR by Castells et al. [46] in the same country using mostly the same sample set (72.1% and 59.9%, respectively). In our study, this frequency is probably underestimated considering that the detection limit and the sensitivity of the antigen-capture ELISA are lower than RT-qPCR, mainly in subclinical infections, and neutralizing antibodies derived either from colostrum or active immune responses may interfere with viral detection by antigen-capture ELISA [47]. Given the high frequency of these pathogens,

transmission routes within and between farms need to be further studied locally to better understand their epidemiological cycle. Rotavirus and *Cryptosporidium* spp. are highly resistant to environmental conditions; calves get infected by contact with feces from dams, which shed these pathogens subclinically contaminating the udder or calving areas. As calves are the main biological amplifyers of these enteric pathogens, transmission between calves occurs by direct contact in communal pens, or indirect contact by fecal contamination of rearing utensils [48]. Waterborne transmission seems plausible, considering that water is the main transmission route for *Cryptosporidium* spp. and has also been suggested for group A rotavirus in dairy calves in Uruguay [49], and that most dairy farms in the country administer untreated underground water or surface water to livestock. Given the geographic proximity of the dairy calf rearing areas and natural surface watersources in Uruguay, the dense network of rivers and the relatively high annual rainfalls and occasional flooding events in this country, calves pose a risk for surface water contamination with fecal pathogens, notably *Cryptosporidium* spp. [50]. Because some subtypes of *Cryptosporidium parvum* are zoonotic and cattle are reservoir of potentially zoonotic strains [51, 52], and considering that *Cryptosporidium* spp. has been identified as a cause of diarrhea in children in Uruguay [53], we further speciated and subtyped the cryptosporidia detected in calves in this study. Interestingly, of seven *C. parvum* subtypes detected in 166 calves, five subtypes detected in 143 calves from nearly all farms had been detected in humans elsewhere and have zoonotic potential [50].

Regarding coinfections and interactions between *Cryptosporidium* spp. and rotavirus, both agents were associated with diarrhea in calves that had individual infections with either pathogen, and in those that were coinfected. However, an unexpected finding was that in coinfected calves the odds of diarrhea were lower than in those infected with either pathogen. Because *Cryptosporidium* spp. and rotavirus are both intracellular pathogens that invade and affect the same target cells (superficial enterocytes) of the small intestine [54, 55], resulting in similar lesions, it can be speculated that they occupy the same cellular or subcellular niches (i.e., receptors, signaling pathways), and/or that infection with one of them somehow interferes with the ability of the other to cause further intestinal damage and diarrhea. A study in mice experimentally infected with *C. parvum* and a strain of *Enterococcus faecalis* administered as a probiotic demonstrated that when both agents were present in the same intestinal location, the bacterium interfered with *C. parvum* infection [56].

In humans, astroviruses cause acute infantile diarrhea [57]; however, the clinical relevance of enteric astroviruses in cattle is not entirely clear. A recent review on viral enteritis in calves concluded that it is currently unclear whether BoAstV is a relevant primary pathogen, a potential cause of disease with coinfections or a clinically irrelevant virus [15]. To the best of our knowledge, ours is the first work in which the possible role of BoAstV was evaluated as one of the agents of NCD and calf mortality in a farm-matched case-control study considering multiple etiologies in the study design and statistical analyses. Despite the relatively high frequency of BoAstV infection (21.7% of the calves and 77.8% of the farms), no association with diarrhea or disease was observed in our study, as suggested by Sharp et al. in Scotland [58]. Conversely, in our study, the frequency of BoAstV infection as an individual agent was significantly higher in non-diarrheic than diarrheic calves (27 of the 31 calves that were only infected with BoAstV were non-diarrheic). This not only calls into question the causative role of enteric BoAstV in NCD, but also suggests a possible beneficial infection with a protective effect on diarrhea which should be further explored. Recent molecular studies by our group indicate a high genetic diversity for BoAstV infecting dairy calves in Uruguay, including three different *Mamastrovirus* species, the most frequent of which represented an unclassified species [59]. Whether cattle harbor *Mamastrovirus* species with zoonotic potential, or whether enteric BoAstV share similarities with a neurotropic BoAstV recently identified as a cause of encephalitis in cattle in this country [60] needs further investigation.

Although the role of BCoV and BVDV as causes of diarrhea in cattle is well documented [13, 14, 61], these viruses were not associated with NCD in our study. The lack of association may have been related to the low frequency of detection, as indicated in other works carried out in various countries [19–21]. However, it should be considered that both agents can cause either enteric (BCoV and BVDV) or systemic disease (BVDV) in older cattle. We have occasionally diagnosed diseases and mortalities caused by BVDV in 3- to 4-month-old heifers [61] and BCoV in neonate calves (unpublished data) in dairy cattle in Uruguay through pathologic examinations and molecular virology, and also detected BVDV in aborted dairy fetuses [61]. In our study, 1.5% of the calves and 18.5% of the farms were positive for BCoV, and 1.3% of the calves and 14.8% of the farms were positive for BVDV, demonstrating the circulation of these viruses at an early age in dairy calves. However, Castells et al. 2019 reported a higher detection rate (7.7%) of BCoV in feces of neonate calves by RT-qPCR [62]. As with rotavirus, BCoV antigen-capture ELISA has a lower sensitivity and limit of detection than RT-qPCR, mainly in subclinical infections, and neutralizing antibodies derived from colostrum or active immune responses may interfere with viral detection by antigen-capture ELISA [47, 63]. Thus, the frequency of BCoV detection in our study is probably underestimated; in this context, the lack of association between BCoV and diarrhea could have been a consequence of the low detection frequency. Regarding BVDV, the relatively low frequency of detection at the calf level was not unexpected, as BVDV rarely causes diarrhea in neonatal calves under field conditions.

Additionally, it should be mentioned that the antigen-capture ELISA performed to detect BVDV in 136 of the 480 calves analyzed for this virus, is not suggested to detect BVDV in sera from persistently infected calves, mainly those younger than 3 months of age in which specific colostrum-derived antibody titers are moderate or high [64, 65]. However, considering that 14.8% of the farms had at least one BVDV positive calf either by antigen-capture ELISA or RT-qPCR, and that BVDV can be particularly responsible for severe economic losses [66], further investigations are needed to assess the impact of this virus to the local livestock sector.

Salmonella enterica was detected in 3.9% of the calves (14 cases and 7 controls) and 29.6% of the farms in this study, these proportions are probably underestimated considering the relative low sensitivity and high specificity of the selective culture for this agent [67]. Even though the proportion of infected animals was more than double in diarrheic versus non-diarrheic calves, the agent was not statistically associated with diarrhea. However, *S. enterica* infection was associated with the presence of fibrin in the feces of the calves. *Salmonella enterica* causes severe intestinal lesions, as well as invasive/septicemic infections leading to death [14]. The intestinal damage induced by this bacterium can be so severe to result in fibrin exudation into the intestinal lumen (fibrinous/necrotizing enteritis/enterocolitis) [68], even without or before manifestation of diarrhea. The presence of fibrin in the feces is suggestive, though not exclusive, of enteric salmonellosis, and should prompt the veterinary practitioners to stablish an early medical treatment to avoid calf mortality.

The association between rotavirus infection and the presence of fibrin in feces in our study was unexpected. From a pathologic standpoint this virus causes superficial enterocyte lysis and exfoliation in the small intestine (mostly jejunum and ileum) resulting in shortening, bluntin and fusion of the intestinal villi (atrophic enteropathy) [14], which is unlikely to result in significant extravasation of fibrin from the propial blood vessels into the intestinal lumen, unless there are secondary bacterial complications leading to ulceration. We did not find multicollinearity problems or confusion bias between rotavirus and *S. enterica* infection and the presence of fibrin in feces based on the statistical test parameters, suggesting that this is not an spurious association between these two pathogens. However, other bacterial pathogens that may cause severe intestinal, colonic and/or cecal damage such as attaching and effacing -enteropathogenic and enterohemorrhagic- *E. coli* , were not assessed for in this study. A study on virulence genes of *E. coli* isolated from diarrheic and non-diarrheic dairy calves in Uruguay, using samples of the dairy calves of this study, found a poor representation of genes associated with the shiga toxin-producing *E. coli* (STEC) / enterohemorrhagic *E. coli* (EHEC) group, as well as enteropathogenic *E. coli* (EPEC) [69]. Interestingly, a study on postmortem findings and laboratory-based diagnosis of causes of death in dairy calves in the USA found that calves with necrotizing and ulcerative intestinal lesions were more likely to be diagnosed with rotavirus infection [70].

All diarrheic *S. enterica*-positive calves that died before weaning did so within 3 days of sampling at ages that ranged between 9 and 18 days, while non-diarrheic *S. enterica*-positive calves, all of which survived after weaning at least until day 122 of age (101 days after sampling, data not shown). The diarrheic *S. enterica*-infected calves in our study had a significantly higher risk of dying during the preweaning period than the diarrheic calves not infected with this agent. Thus, salmonellosis should be considered a significant cause of calf mortality in the rearing period in dairy farms in Uruguay. Besides its impact on animal health, the role of cattle as sources of human salmonellosis should be further studied, as *S. enterica* has been recognized as a human pathogen in this country [53, 71], where the cattle population per capita is the highest in the world [72]. The predominant *S. enterica* serotype in our study was *S.* Typhimurium (19 calves in 7 farms), followed by *S.* Anatum (2 calves from the same farm). Because antibiotic resistance in animal and human pathogens is of major global concern and multi-drug-resistant *S.* Typhimurium strains have been identified in human patients in Uruguay [71], we assessed antibiotic susceptibility of all the *Salmonella* strains obtained in this study [73]. The minimum inhibitory concentration to 14 antibiotics in 9 antibiotic classes was assessed by microdilution. All 21 strains were resistant to at least one antibiotic class, and 11/21 strains were resistant to ≥ 3 antibiotic classes (predominantly tetracyclines, aminoglycosides and beta-lactams), and were thus considered multi-drug resistant strains [73]. The phenotypic and molecular bases for antibiotic resistance need to be further explored.

E. coli F5+ was found in a relatively low frequency at the individual (2.1% of the calves) and farm (18.5%) levels and was not associated with NCD. The low frequency of *E. coli* F5+ in neonatal calves had been previously documented in the region [74, 75], as well as in other parts of the world [12, 20, 21]. It should be considered that enterotoxigenic *E. coli* affects calves < 1 week of age [76], and our sampling frame included calves up to 4 weeks of age, which probably represents a bias. In our study, of the 11 calves that tested positive for *E. coli* F5+, 9 were ≤ 5 days of life and the remainder two were 12 and 15 days old. No association with diarrhea was found even when only calves within the first week of life were considered for the statistical analysis (not shown). Furthermore, it should be considered that even though the expression of the fimbrial antigen F5 and the production of heat-stable toxin (STa) are highly associated in enterotoxigenic *E. coli*, some studies have found *E. coli* F5+ strains without toxigenic potential (i.e., PCR negative

for the STa-encoding gene). This could explain why diarrhea was not observed in some of the positive calves [77–80]. In addition, it is not unexpected to have false negative results using antigen-capture ELISA for F5, as this test has a low sensitivity [12].

Determining the STP concentration in serum is an indirect way of assessing the immune status of the calves, particularly the transfer of passive immunity (colostral antibodies) in the first week of life [23, 38]. Low concentrations of STP have been associated with increased morbidity and mortality [5, 28]. In our study, almost half of the calves (47.4%) sampled for STP determination had values < 5.6 g/dl consistent with FTPI. A higher concentration of STP was observed in non-diarrheic calves, as well as in calves that were negative for rotavirus. Calves with higher STP concentrations had lower odds of manifesting diarrhea as well as being positive for this pathogen. This suggests that higher STP concentrations, indicative of successful transfer of passive immunity, may have had a protective effect against diarrhea and rotavirus infection. Vaccines available to prevent NCD usually contain rotavirus and other various viral and bacterial antigens (sometimes including BCoV and *E. coli* F5+, depending on the manufacturing laboratory) and aim at increasing specific colostral immunity in the dams, so they require adequate colostrum management practices to warrant successful transfer of immunity to protect the calves. Whether rotavirus antigens included in the vaccines available in Uruguay (all of which consist of inactivated virus) protect against the predominant viral strains needs to be addressed and deeper antigenic characterizations of local rotavirus strains should be performed. Currently, there are no commercially available vaccines for *Cryptosporidium* spp.; however, it has been postulated that calves with an adequate immune status, acquired through colostrum, are less likely to shed this agent in feces [81] and to have clinical cryptosporidiosis [82]. Interestingly, in our study, the mean STP concentration was higher in *Cryptosporidium* spp.-negative (5.71 g/dl, $n = 66$) than -positive calves (5.47 g/dl, $n = 29$) (data no shown). Although this difference was not statistically significant, these mean STP concentrations were above and below, respectively, of the cutoff value to determine FTPI [38]. This highlights the importance of applying readily available and cost-effective management practices, such as vaccination of the dams to obtain quality colostrum and its early administration to calves to prevent NCD. Although vaccination to prevent NCD is recommended in all dairy farms, it is most meaningful in those where colostrum management practices are adequate. Unfortunately, inadequate colostrum management practices are widespread among dairy farms in Uruguay [4], which may partially explain the high percentage of calves with low STP concentrations found in our study. Regarding the lack of statistical association between STP and preweaning death, it should be noted that among 31 calves with STP values < 5.6 g/dl, 14 had missing data on preweaning death. In addition, 22 calves with STP values ≥ 5.6 g/dl had missing data on preweaning death (Supplementary Material 1). This represents 37.9% (36/95) of calves with missing data, which probably influenced the outcome of the statistical analysis.

Conclusions

We generated reliable epidemiological information to apply specific control and preventive measures to reduce NCD associated losses in Uruguay. This was achieved through a farm-matched case-control study, evaluating multiple enteropathogens, clinical signs, preweaning death, FTPI, and their associations. We conclude that NCD is an important cause for mortality of dairy calves in Uruguay, regardless of its cause. *Cryptosporidium* spp. and rotavirus cause NCD and are frequent both at the calf and farm levels. *Salmonella enterica* infection results in fibrinous stools and increases the risk of preweaning mortality in diarrheic calves. BoAstV, despite being frequent, is not a primary cause of diarrhea for dairy calves in Uruguay. Although the frequencies of BCoV, BVDV and *E. coli* F5+ are relatively low, these pathogens are probably underestimated and their role in neonatal disease should not be disregarded. The STP concentration in neonate calves has a protective effect against diarrhea and rotavirus infection, demonstrating the importance of applying adequate colostrum management practices to improve neonatal calf health and well-being and reduce diarrhea-associated mortality. Lastly, neonatal dairy calves in Uruguay are reservoirs of potentially zoonotic pathogens, notably *Salmonella* Typhimurium and *Cryptosporidium* spp. that have been identified in human patients in this country.

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Code availability Not applicable

Author contribution RDC, MF, FRC, and FG conceived the study. RDC, MLC, and COS performed field work. RDC, MLC, COS, MC, LM, and RC conducted laboratory testing. RDC and LGC analysed data. RDC and FG wrote the first draft and final version of the manuscript. All authors read, edited, and approved the final version of the manuscript.

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Data availability The datasets generated for this study are available as Supplementary Material 1.

Declarations

Ethics approval Procedures involving sampling of calves were reviewed and approved by INIA's animal ethics committee for the use of animals in experimentation (protocol #20199).

Consent to participate All authors gave their consent to participate.

Consent for publication All authors gave their consent for the publication of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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Capítulo 6_ Manuscrito 6:

Maya L, Panzera Y, Pérez R, Marandino A, Colina R. GENOME SEQUENCES OF NOVEL BVDV-1a URUGUAYAN STRAINS.

Este trabajo tuvo como cometido aislar y secuenciar el genoma completo de 2 cepas uruguayas BVDV-1a.

Los principales resultados de este trabajo son los siguientes:

- Se aislaron los primeras cepas Uruguayas de BVDV
- Se secuenciaron los primeros genomas completos de cepas uruguayas de BVDV
- Las distancia genética entre las cepas de campo y vacunal de BVDV estudiadas previamente con regiones parciales del genoma viral, fue confirmada al realizar los análisis comparativos usando el genoma completo
- La similitud de las cepas uruguayas BVDV-1a y la cepa de origen Chino G5S fue confirmada
- Los protocolos para el aislamiento y secuenciación de genomas de BVDV fueron puestos a punto y podrán ser utilizados posteriormente para realizar más aislamientos de este subtipo viral, así como también del subtipo BVDV-2b que también circula en nuestros rodeos

GENOME SEQUENCES OF NOVEL BVDV-1a URUGUAYAN STRAINS

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a pathogen of economic importance that affects production and reproduction in cattle. BVDV has been associated with reproductive failure, abortion, and respiratory and enteric infections. BVDV is endemic in Uruguay and most herds (~80%) are seropositive to the virus.

The BVDV-1a subtype is prevalent in Uruguayan territory; this subtype has diverged locally and is distantly related to the NADL vaccine strain. The amino acid differences between field and vaccine viruses may lead to failure in neutralization/vaccine protection.

In the present study, two Uruguayan BVDV-1a strains were isolated by cell culture and their complete genomes were obtained using Illumina sequencing. These isolates were the first obtained in Uruguay and became the starting point for building a Biobank of BVDV strains. Comparative analyzes of the genomes of the Uruguayan strains revealed that are genetically closely related to GS5 Chinese strain, and diverge from the NADL vaccine strain. The most notable differences that were found among vaccine and field strain were found on the encoding sequences of E2 membrane glycoprotein, Core and NS4. Notably, we found that most of these mutations leads to synonymous changes on Core and NS4 proteins; while on E2 membrane glycoprotein most changes were non-synonymous. These findings support and will allow more genetic and evolutionary studies, including more in-depth comparative analyzes among field and vaccine strains to raise the question of whether current vaccines need to be reformulated in order to be massive protective.

Bovine viral diarrhea virus (BVDV) is a pathogen of global economic importance that causes a wide variety of clinical signs and reproductive failure in cattle [Houe 1999]. BVDV belongs to the genus *Pestivirus* in the family *Flaviviridae* and has a single-stranded positive-sense RNA genome of approximately 12.3 kb in length flanked at both ends by untranslated regions (UTRs). The only open reading frame (ORF) of BVDV encodes a single polyprotein of 3898 amino acid residues that is cleaved co- and post-translationally into 11-12 proteins (Meyers, 1996).

There are three BVDV species currently recognized by the International Committee on Taxonomy of Viruses (ICTV), namely BVDV-1, BVDV-2, and HoBi-like Pestivirus (ICTV).

Uruguay is a livestock country, and agro-industrial exports represent 8.4% of the country's gross domestic product (MGAP, DIEA 2018). Uruguay has approximately 12 million cattle, including beef and dairy cattle and this number has remained practically unchanged since 1998. BVDV is presumably one of the factors contributing to reduce pregnancy and calf survival at weaning (PLANISA). BVDV is endemic in Uruguay; the 98.8% of the farms were seropositive for BVDV according to a sampling study performed by the Uruguayan livestock ministry in 2015. The BVDV seroprevalence was 80.9% and 82.8% for dairy farms and beef cattle, respectively. In Uruguay there is not sanitary program to BVDV and its vaccination is not compulsory; only 32.1% of dairy farms and 14.6% of beef cattle farms vaccinate against BVDV (Dr. Federico Fernández, Ministerio de Ganadería, Agricultura y Pesca, MGAP, personal communication). There are reports of BVDV acting on reproductive failures, abortions, and BVDV associated diseases, including mucosal disease and respiratory and enteric problems, due to co-infections associated with bacteria (Maya et al., 2016; Macías-Rioseco et al., 2020; da Silva Silveira et al., 2020).

BVDV-1a is the major subtype in Uruguayan herds [Maya et al., 2020]. Previous studies using partial nucleotide sequences of 5´UTR, N^{pro} and E2 genomic regions revealed that BVDV-1a Uruguayan strains are genetically distant from NADL vaccine strain and others worldwide BVDV-1a strains, having some amino acids changes that could lead to failure of protection through vaccination (Maya et al., 2020).

In the present study, 2 Uruguayan BVDV-1a strains were isolated from serum samples and their complete genomes were sequenced and analyzed.

Briefly, serum samples of strains 754UYAFA4/112015 and 2402UYSJ/2016 were subjected to virus isolation using Pestivirus-free Madin-Darby bovine kidney cells (MDBK) with Minimum essential medium (MEM) supplemented with 10% horse serum and antibiotics (Penicillin (10,000UI/mL), Streptomycin (10mg/mL), Amphotericin B (250µg / mL)). The plates were incubated at 37 °C with 5% CO_2 and examined daily for up to 3 days. Three consecutive blind passages were performed for each sample.

MDBK cells infected with 754UYAFA4/112015 and 2402UYSJ/2016 strains were submitted to three freeze/thaw cycles to release BVDV viral particles. After that, viral stocks were centrifuged at 3000 × rpm for 3 min to clarify the supernatant of cellular debris before RNA isolation. RNA was isolated using 1 mL of cultured supernatant using Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA). The extracted RNA was reverse-transcribed using the Maxima H Minus Double-Stranded cDNA Synthesis kit (Thermo Fisher Scientific, USA). The cDNA was purified using AMPure XP (Benchman, USA) and 100 ng was subjected to Nextera DNA Flex Library Preparation kit (Illumina, USA) using MiniSeq Mid Output Reagent Cartridge (300-cycles, paired-end reads). Library was sequenced on an Illumina MiniSeq platform. Adapter/quality trimming and filtering of raw data were performed with BBDuk and clean reads were mapped to the BVDV-1a strain G5S isolated in China in 2013 (KJ541471) as reference sequence using Geneious Prime 2020.1.2 (https://www.geneious.com/). The complete genomes of 754UYAFA4/112015 and 2402UYSJ/2016 strains were submitted in GenBank database under accession number x and x, respectively.

Genetic similarity between the two Uruguayan strains and the NADL strain was analyzed using MEGA 7 (Kumar, Stecher and Tamura, 2016).

A phylogenetic tree was constructed using the maximum likelihood (ML) method, and the statistical significance were conducted using the bootstrap method (1000 repetitions) in MEGA 7 (Kumar, Stecher and Tamura, 2016). The model of nucleotide substitution that best fit the dataset (GTR + gamma) was selected using the jModelTest program according to the Akaike information criterion (AIC; Akaike, 1974) (Posada, 2008). The phylogenetic tree included Uruguayan sequences and 75 sequences of BVDV-1 species available on the NIAID Virus Pathogen Database and Analysis Resource (ViPR) through the web site at https://www.viprbrc.org/brc/vipr_genome_search.spg. Redundant, shorter, and

not subtyped genomes were removed from the final data set. Border disease virus (BDV) reference sequence (X818) was used as outgroup.

Using the UGENE software, an ORF of 11,694 nucleotides and a polyprotein of 3898 amino acid residues in length were identified in both Uruguayan strains, and 11 individual proteins were found that were annotated (Table 1).Nucleotide and amino acid similarities among Uruguayan strains were 94,7%, and 96,7%, respectively (Table 1).

Uruguayan strains associate with the BVDV-1a strains on phylogenetic analysis and appear closely related to the Chinese strain GS5 (Figure 1, black dots). Nucleotide and amino acid similarities between the Uruguayans and GS5 strains ranged between 92%, and 95%, respectively. In contrast, Uruguayan strains were distantly to NADL vaccine strain on phylogenetic tree (Figure 1, asterisk), having a nucleotide and amino acid divergence of 12% and 8%, respectively (Table 1). Nucleotide and amino acid divergence of the Uruguayan and NADL strains is higher in variable genomic regions such as the encoding region of E2 membrane glycoprotein (Table 1) (El Omari et al., 2013; Maya et al., 2020). Another genomics regions such as encoding region of core (capsid protein), and NS4A (cofactor for NS3 serine protease) showed higher divergence at nucleotide level than the encoding region of E2 membrane glycoprotein (showed in bold on Table 1) (Murray et al., 2008; Xu et al., 1997). When those genomic regions were compared at amino acid level its variability decreased drastically, showing that most of the nucleotide changes were non- synonymous (Table 1). It is likely that genomic regions (e.g., E2 encoding region) were subjected to positive selective pressures exerted by the host's immune system (Ma et al., 2018). These regions would evolve faster by the acquisition of non-synonymous changes, explaining the high amino acid divergence in the E2 membrane glycoprotein compared with the remaining viral proteins (Table 1)

The genetic variability of circulating BVDV strains it could question the efficacy of current available vaccines and the sensitivity of diagnostic BVDV tests. Thus, the present study complement and adds remarkable and valuable information to the previous studies of BVDV Uruguayan strains that our group has been doing for the last 5 years (Maya et al., 2016, 2020).

Strains 754UYAFA4/112015 and 2402UYSJ/2016 were the first Uruguayan autochthonous isolates, providing a new framework to understand BVDV genome

variability of Uruguayan strains and to propel BVDV applied research in Uruguay. Our results represent the milestone in the construction of a collection of Uruguayan strains of BVDV that will allow more genetic and evolutionary studies, including more in-depth comparative analyzes among field and vaccine strains. The knowledge acquired in this study it is also valuable to continue BVDV surveillance, keeping the diagnostic tests up-to-date, develop massive and effective vaccines, and promote more effective BVDV mitigation strategies.

Table 1. Nucleotide and amino acidic percentages identities between 754UYAFA4/112015 and 2402UYSJ2016, and NADL vaccine strain

Protein name and Nucleotide position in the genome	Npro 368-863	Core 864-1169	Ems 1170-1850	E1 1851-2435	E2 2436-3557	P7 3558-3767	NS23 3768-7175	NS4A 7176-7367	NS4B 7368-8408	NS5A 8409-9896	NS5B 9897-12056	CDS
754UYAFA4/112015	86,7%	83%	88%	88,7%	85,8%	86,2%	87,9%	81,3%	90%	85,7%	88,8%	87,8%
2402UYSJ/2016	88,9%	83,7%	88%	87,5%	84,7%	84,8%	88,5% inserción 270nt, 90aa	85,9%	89,3%	85,5%	89,5%	88%
Amino acidic length of each protein	Npro 168	Core 102	Ems 227	E1 195	E2 374	P7 70	NS23 1130	NS4A 64	NS4B 341	NS5A 496	NS5B 719	CDS
754UYAFA4/112015	90,5%	89,2%	93,4%	90,8%	86,6%	87,1%	94,3%	95,9%	90,8%	88,3%	92,3%	92,2%
2402UYSJ/2016	94%	89,2%	95,1%	89,7%	84,5%	88,6%	94,5%	93,8%	90%	88,5%	92,3%	92,2%

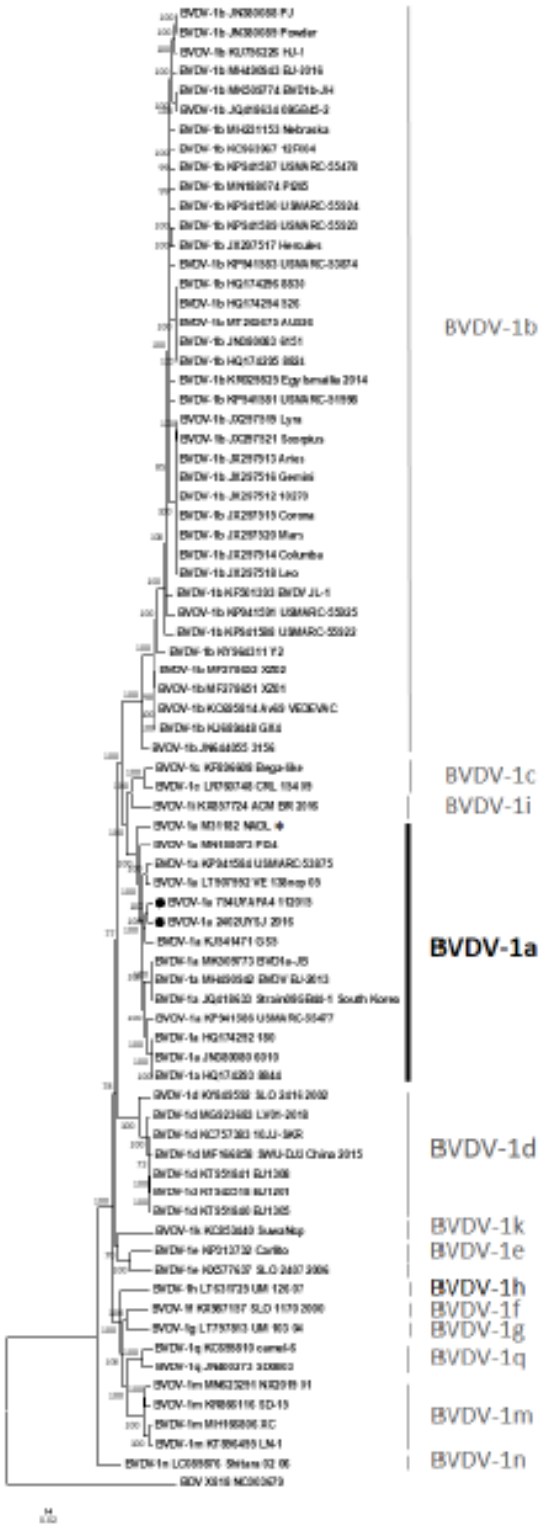

Figure 1. Phylogenetic analysis of full encoding sequences of BVDV strains. Uruguayan strains are indicated by black dots and NADL vaccine strain by an asterisk. BVDV-1 subtypes are shown on the right side of the figure, and BVDV-1a subtype is highlight in bold. Numbers at the branches of the trees are bootstrap values. A BDV (border disease virus) sequence was included in the analysis as an out-group.

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5- DISCUSIÓN GLOBAL

Uruguay es un país ganadero y la economía del país depende en gran medida de la producción ganadera. En el año 2018 alrededor del 27% de las exportaciones que realizó Uruguay correspondieron a carne bovina y bovinos en pie, representando alrededor de 2 millones de dólares (Anuario estadístico DIEA 2019).

BVDV es un virus con gran impacto económico ya que afecta la reproducción y producción bovina. BVDV, de acuerdo con los datos de relevados en un muestreo del año 2015, el 80.9% del ganado de leche y el 82.8% del ganado de carne es seropositivo a BVDV en Uruguay (Dr Federico Fernández, Ministerio de Ganadería, Agricultura y Pesca, MGAP, comunicación personal). Uruguay no tiene un plan sanitario para el control de BVDV y la vacunación no es obligatoria, quedando a criterio del productor o del médico veterinario del establecimiento.

En rodeos en que BVDV es endémico se estima que las pérdidas económicas oscilan entre 8.4 y 113 dólares/vaca/ año; y en rodeos que presentan brotes de BVDV las pérdidas van desde 37 a 3090 dólares/animal/año (Yarnall and Thrusfield, 2017). Las mayores pérdidas económicas se deben a afecciones reproductivas. Las repercusiones económicas de BVDV como fruto de la inmunosupresión y el menor rendimiento lechero son menos estudiadas, por lo que se cree que están subestimadas (Yarnall and Thrusfield, 2017).

Países ganaderos como Escocia, Nueva Zelanda y Suiza, han implementado exitosamente estrategias para el control de BVDV, basados en vigilancia continua del virus, vacunación y eliminación de animales PI. De esta manera han visto mejoras en la reproducción, rendimiento lechero, y se redujeron las enfermedades asociadas a BVDV, de manera que han podido mitigar las pérdidas económicas debidas a este patógeno (Oguejiofor *et al*., 2019).

En Uruguay, de acuerdo con los puntos planteados en el PLANISA, antes de elaborar una estrategia para el control y la mitigación de BVDV de nuestros rodeos, teníamos un "camino" previo por recorrer, y es ese el punto donde empieza esta tesis.

Dados los antecedentes y las necesidades respecto a la temática BVDV planteada por el PLANISA, lo primero que realizamos fue desarrollar una metodología molecular robusta para la detección de BVDV.

Pusimos a punto un método de extracción de ácidos nucleicos que adecuamos a todos los tipos de matrices recibidas: suero, sangre, semen, leche y diversos tipos de tejidos. Para la detección molecular de BVDV, adecuamos el protocolo de PCR en tiempo real (qPCR) previamente desarrollado por Hoffman *et al*., (2006) y Gaede *et al*., (2005) que amplifica 207 pb de la 5´UTR del genoma de BVDV, modificando algunos nucleótidos por degeneración para hacer posible la detección de BVDV-1, BVDV-2 y el Pestivirus tipo HoBi (Tabla 1, manuscrito 1 Maya *et al*., 2016). En la

detección de BVDV, la qPCR es más sensible que el ELISA de captura IDEXX BVDV Ag/Serum Plus (IDEXX- Switzerland) (Hanon *et al*., 2014; Maya *et al*., 2016).

5.1- Diversidad genética de BVDV en Uruguay

Para estudiar la diversidad genética de BVDV por medio de árboles filogenéticos, la 5´UTR es la región genómica más comúnmente usada. Esta región no codificante del genoma de BVDV, es corta y altamente conservada, permitiendo diferenciar las especies de BVDV, pero no es suficientemente informativa para la correcta asignación en subtipos virales (Xia *et al*., 2007; Booth *et al*., 2013). Para estudiar la diversidad genética de las estirpes de BVDV que circulan en Uruguay, por medio de árboles filogenéticos con nodos de buen soporte estadístico, se utilizó un fragmento de 607 pb de las secuencias nucleotidicas concatenadas de la 5´UTR y la proteasa viral N^{pro} (5´UTR/N^{pro}), y el extremo amino terminal de la glicoproteína de membrana E2 (Maya *et al*., 2016, 2020).

De esta manera detectamos las especies BVDV-1 y BVDV-2, y los subtipos virales de BVDV en los siguientes porcentajes: BVDV-1a (87.5%), BVDV-1i (6.2%), y BVDV-2b (6.3%) (Maya *et al*., 2016). Al aumentar el número de casos positivos a BVDV (n= 42) en nuestros siguientes trabajos (Maya *et al*., 2020; da Silva Silveira *et al*., 2020), observamos que la epidemiología molecular de BVDV permaneció incambiada y BVDV-1a continuó siendo el subtipo viral mayoritario, seguido por los subtipos virales BVDV-2b, y BVDV-1i.

El subtipo BVDV-1a fue detectado en el 81% de los animales positivos. En nuestro primer artículo, analizamos 12 secuencias de la región genómica 5´UTR/N^{pro} y en el segundo artículo el número ascendió a 28 cepas BVDV-1a. Al analizar un mayor número de cepas observamos una diversificación geográfica del subtipo BVDV-1a en Uruguay, formando 2 linajes Uruguayos a los que denominamos como BVDV-1a linaje1 uy y BVDV-1a linaje2 uy (Figura 1, manuscrito 3 Maya *et al*., 2020). El BVDV-1a linaje1 uy está compuesto por 24 cepas Uruguayas y se agrupa con cepas de Brasil y 1 cepa de origen Chino. El BVDV-1a linaje2 uy, integrado por un menor número de cepas (n= 4), se agrupa también con una cepa de origen Chino.

De total de animales positivos a BVDV, un único animal muestreado en el año 2014 estaba infectado con el subtipo **BVDV-1i** (Figura 1, manuscrito 1 Maya *et al*., 2016). El hecho que sea un único caso, podría estar denotando que este subtipo no tendría una circulación extensa en nuestros rodeos, y su hallazgo representaría un hecho aislado. El subtipo BVDV-1i fue descrito por primera vez en 1999 en el Reino Unido (Vilcek *et al*., 2001), y su incidencia fue aumentando (Strong *et al*., 2013). Este subtipo viral parecía estar restringido a Reino Unido hasta que en el año 2014 fue detectado en Uruguay y posteriormente en el año 2016 en Brasil (Maya *et al*., 2016; Mósena *et al*., 2016). Las

cepas de Uruguay y Brasil se relacionan más cercanamente entre sí, y se distancian de las cepas BVDV-1i de Reino Unido (Figura 1, manuscrito 3) (Mósena *et al*., 2016; Maya *et al*., 2020).

En Uruguay el subtipo BVDV-2b, de acuerdo con nuestro muestreo, no parece tener alta incidencia representando el 16.6% de total de animales positivos. En las 3 muestras que pudimos amplificar las regiones genómicas 5´UTR/N^{pro} y hacer análisis comparativos, observamos que las cepas Uruguayas 2391UYRN/2016 y 2769UYRN/2016 se agruparon, y se separaron del resto de las cepas de este subtipo viral, incluida la cepa Uruguaya 439RvUY/082014 (Figura 1, manuscrito 3). Esto podría estar denotando una diversificación del subtipo BVDV-2b en nuestro territorio, pero sería apropiado aumentar el número de cepas para estar en condiciones de afirmar este comportamiento (Maya *et al*., 2020).

Los subtipos virales descritos anteriormente fueron detectados en predios con fallas reproductivas, y fueron vinculados como agente infeccioso causante o co-participante en abortos (Tabla 1, manuscrito 2) (Maya *et al*., 2016; Macías-Rioseco *et al*., 2020; da Silva Silveira *et al*., 2020). Estos subtipos virales, además fueron detectados en otras enfermedades asociadas a BVDV como ser enfermedad de las mucosas, y en problemas respiratorios y entéricos co-infectando de manera asociada a bacterias y otros virus (Tabla 1, manuscrito 2; Tabla 1 manuscrito 5) (Macías-Rioseco *et al*., 2020; da Silva Silveira *et al*., 2020; Caffarena *et al*., 2021). El reconocer e identificar las enfermedades asociadas a BVDV es fundamental para la vigilancia de BVDV y una pieza esencial en la elaboración de programas de control.

5.2- Caracterización genética y evolutiva de las cepas de BVDV-1a Uruguayas

Las cepas del subtipo BVDV-1a, siendo el subtipo mayoritario en nuestros rodeos, ameritaron a ser analizadas en mayor profundidad.

Los linajes BVDV-1a linaje1 uy y BVDV-1a linaje2 uy surgen en Uruguay como producto de la evolución que ha tenido este subtipo viral en nuestros rodeos. De acuerdo con nuestros cálculos de TMRCA (Time Most Recent Common Ancestor), BVDV-1a comenzó a circular en Uruguay en 1990 (Tabla 4, manuscrito 3). Estos resultados concuerdan con los datos clínicos, que BVDV circularía en Uruguay desde los 80´, y en 1996 BVDV fue detectado por primera vez por inmunohistoquímica (Saizar *et al*., 1998).

En estos años luego de su emergencia en nuestro territorio, BVDV-1a ha usado a la mutación puntual como mecanismo de evolución y generador de variabilidad genética. De esta manera, BVDV-1a ha acumulado mutaciones a una tasa de $3.48x10^{-3}$ sustituciones nucleotídicas/sitio/año (Tabla 4, manuscrito 3), que le han permitido adquirir características genéticas propias, que han diferenciado

las cepas BVDV-1a Uruguayas de otras cepas BVDV-1a, formando los linajes uruguayos ya mencionados.

En particular las cepas de los linajes BVDV-1a Uruguayas se han diversificado de la cepa vacunal NADL (Tabla 2, manuscrito 3). Al analizar la proteasa viral N^{pro} y la glicoproteína de membrana E2 se encontraron cambios amino acidícos, 4 y 11, respectivamente (Tabla 3, manuscrito 3). Era de esperar encontrar más cambios amino acídicos en la proteína E2 dado que es el principal sitio antigénico del virión por su rol de unirse al receptor celular en la célula hospedera. De las sustituciones aminoacídicas encontradas en E2 son de destacar las de los residuos 71, 87, 88 y 90, dado que forman parte de 2 epitopes en E2 que pueden producir una falla de neutralización y protección en animales vacunados con la cepa NADL (Figura 4, manuscrito 3) (El Omari *et al*., 2013; Ridpath, 2013). El linaje BVDV-1a linaje1 uy tiene 4 cambios en los amino ácidos $T_{38}V_{54}T_{68}E_{81}$ en E2 que parecen ser únicos de las cepas Uruguayas de este linaje, constituyendo lo que denominamos como "su huella dactilar", que lo diferencian del linaje BVDV-1a linaje2 uy ($M_{38}M_{54}I_{68}K_{81}$) y de la cepa NADL ($K_{38}M_{54}I_{68}K_{81}$), y de las restantes cepas BVDV-1a incluidas en el análisis (Tabla 3b, manuscrito 3) (Maya *et al*, 2020). Al momento no hay reportes de que estos aminoácidos formen parte de sitios antigénicos, sin embargo, pueden cambiar las propiedades de la E2 dado que algunas de estas sustituciones implican cambios en las propiedades químicas de los amino ácidos (Maya *et al*., 2020). A futuro haremos análisis para esclarecer si esta "huella dactilar" del linaje BVDV-1a linaje1 uy juega un rol en el fitness de estas cepas Uruguayas.

5.3- Secuenciación del genoma completo de cepas BVDV-1a Uruguayas

La variabilidad genética entre las cepas circulantes de BVDV hace difícil la elaboración de estrategias para el control y mitigación de este patógeno mediante el diseño de tests diagnósticos robustos, y también el desarrollo e implementación de vacunas efectivas. Teniendo en mente esto y dados los conocimientos previos generados de las cepas uruguayas y descriptos en los puntos anteriores, consideramos que el paso siguiente más atinado a realizar en nuestra investigación en BVDV debía ser el aislamiento de cepas uruguayas y la secuenciación de sus genomas completos (manuscrito 6). En este sentido el aislamiento de cepas BVDV-1a uruguayas y la secuenciación de sus genomas completos hacen un aporte importante. Se escogió comenzar este tipo de estudios con las cepas BVDV-1a por las características estudiadas y discutidas anteriormente. Los protocolos de aislamiento y secuenciación masiva fueron puestos a punto para cepas BVDV en general, por lo que luego podremos continuar con el aislamiento y secuenciación de los otros subtipos presentes en nuestros rodeos que también ameritan estudios más profundos.

Al comparar mediante un árbol filogenético los genomas completos de las cepas BVDV-1a uruguayas con 75 genomas de los diferentes subtipos de la especie BVDV-1, se observó que ambas cepas uruguayas se agrupan con la cepa del subtipo BVDV-1a G5S procedente de China (Figura 1, manuscrito 6). Por tanto, el uso de las regiones 5´UTR/N^{pro} y glicoproteína de membrana E2 es válida para la clasificación en especies y subtipos de BVDV ya que de las 2 cepas cuyos genomas fueron secuenciados ya se había observado esta relación cercana con la cepa China G5S en los árboles filogenéticos realizados con las regiones genómicas 5´UTR/Npro (Figura 1, manuscrito 3) y la glicoproteína de membrana E2 (Figura 3, manuscrito 3).

Los genomas de ambas cepas uruguayas son divergentes respecto al genoma de la cepa vacunal NADL tanto a nivel nucleotídico y aminoacídico (Tabla 2, manuscrito 6). Como se discutió anteriormente, divergencia de las cepas uruguayas y vacunal queda más evidente en regiones más variables como la glicoproteína de membrana E2 en comparación con la proteasa viral. Evidentemente la diversidad de estas cepas a lo largo del genoma varía por las diversas presiones selectivas a las que están sometidas a las distintas regiones genómicas, reflejándose que, en promedio, la divergencia nucleotídica y aminoacídica de las cepas uruguayas y vacunales en el genoma completo es menor que cuando se estudió la proteasa Npro y la glicoproteína de membrana E2 (Tabla 2, manuscrito 3).

Es de destacar que estas 2 cepas representan los primeros aislamientos de cepas autóctonas uruguayas. Este representa el primer paso en la construcción de un cepario uruguayo de BVDV que permitirá realizar estudios genéticos y evolutivos de las cepas uruguayas, así como también análisis comparativos de las cepas de campo y vacunales en mayor profundidad una vez que contemos con un mayor número de genomas completos de cepas Uruguayas.

6- CONCLUSIONES

3- Se diseñó un método de detección molecular de BVDV robusto que permite su detección a partir de diferentes matrices

4- Se detectó en los rodeos uruguayos la presencia de las especies BVDV-1, subtipos BVDV-1a y BVDV-1i; y de la especie BVDV-2 el subtipo BVDV-2b

5- En nuestro muestreo no fue detectado el Pestivirus tipo HoBi

6- El subtipo prevalente en nuestros rodeos es BVDV-1a

7- Se realizaron aportes fundamentales para la vigilancia de BVDV en Uruguay y muy importantes para la elaboración de futuros planes de control, identificando y reconociendo enfermedades causadas por BVDV, y a las cuales se encuentra asociado.

8- El subtipo BVDV-1a a partir que empezó a circular en nuestros rodeos hace 30 años apx. Fue acumulando mutaciones puntuales al punto tal que se formaron 2 linajes BVDV-1a uruguayos con características genéticas propias.

9- Las cepas BVDV-1a Uruguayas son divergentes respecto a la cepa vacunal NADL, provocando posiblemente fallas en animales vacunados a nivel de neutralización y protección

10- Las cepas de los linajes Uruguayos BVDV-1a son similares a cepas aisladas en China

11- Estas similitudes y divergencias se observaron al estudiar las regiones genómicas 5´UTR/Npro, la glicoproteína de membrana E2, y fueron confirmadas cuando los estudios comparativos se ampliaron y se utilizó el genoma completo de BVDV.

12- Se realizaron los 2 primeros aislados autóctonos Uruguayos, representando el paso inicial en la construcción de un cepario nacional de BVDV

13- Los 2 genomas de BVDV secuenciados en esta tesis representan los primeros genomas completos de BVDV Uruguayos.

7- PERSPECTIVAS

Creemos que los resultados obtenidos en esta tesis que han sido publicados en revistas internacionales arbitradas, en conjunto con otros trabajos que han publicado otros miembros de este equipo multidisciplinario han aportado información valiosísima y han hecho más visible la temática BVDV en los rodeos uruguayos. Seguiremos trabajando en pos de un plan de saneamiento de BVDV y es por eso que nos planteamos las siguientes perspectivas:

- *Continuar la vigilancia de BVDV.*

Esto nos permitirá mantener actualizada la epidemiología molecular de BVDV en Uruguay y nuestras metodologías de detección molecular de BVDV.

Identificación y remoción de animales PI de manera coordinada con los productores y veterinarios de establecimientos.

Testear animales antes de movilizar ganado de un predio a otro.

- *Generar un cepario nacional de BVDV*

Realizar más aislamientos virales a partir de muestras clínicas con el fin de comenzar a generar un cepario nacional de BVDV datado.

-Realizar ensayos de neutralización con las cepas Uruguayas aisladas

Al momento sabemos que las cepas uruguayas tienen diferencias amino acídicas en 2 epítopes de la glicoproteína de membrana E2, que provocarían una falla en la neutralización por los anticuerpos generados por la cepa NADL. Estos datos teóricos hay que probarlos *in vitro.* Para esto se harán ensayos de neutralización con cepas de BVDV uruguayas aisladas en cultivos celulares.

- *Amplificar un mayor número de genomas de BVDV y explorar nuevas regiones del genoma de las cepas uruguayas*

El genoma de BVDV es variable, lo que provoca que haya una falla en el apareamiento de bases de los cebadores publicados en la literatura y las cepas Uruguayas. Por tanto, al contar con los 2 genomas secuenciados en esta tesis podremos diseñar cebadores específicos para las cepas Uruguayas de BVDV. Esto nos permitirá amplificar y estudiar otras regiones del genoma de BVDV, y a su vez amplificar un mayor número de genomas.

- *Desarrollos biotecnológicos*

La construcción del cepario uruguayo y el estudio en mayor profundidad de los genomas se realizará en vistas de comenzar a trabajar en desarrollos biotecnológicos para lograr generar prototipos de vacunas en conjunto con instituciones de investigación nacionales y regionales.

- *Profundizar colaboraciones con laboratorios de Uruguay y laboratorios de la región*

Nuestro grupo trabaja en colaboración con otros grupos de Uruguay y de la región. Realicé una pasantía en el laboratorio de Virología del referente en BVDV el Dr. Eduardo Flores Furtado ubicado en la Universidad de Santa María de Río Grande del Sur- Brasil. La situación epidemiológica por la pandemia de COVID-19 que vivimos desde el año pasado ha impedido realizar una nueva pasantía, pero ni bien se den nuevamente las condiciones se retomarán trabajos conjuntos en el régimen de pasantías.

En colaboración con la Plataforma Genómica de Facultad de Ciencias dirigida por la Dra. Yanina Panzera secuenciamos los 2 primeros genomas completos de las cepas uruguayas de BVDV. Ni bien la situación epidemiológica lo permita haré una pasantía en la plataforma, como era la idea original hasta que se presentó la actual pandemia, y aprenderé los pormenores de NGS y secuenciaremos más cepas uruguayas.

En este momento nos encontramos en una etapa y grado de avance en la investigación de BVDV en que podemos profundizar estas colaboraciones y establecer nuevas a nivel nacional, y regional.

- *Estudiar BVDV en otras especies*

BVDV infecta principalmente a bovinos, pero también ha sido detectado en otras especies animales de pezuña hendida como ser ovejas y cerdos. En el caso de los ovinos hay predios en los que hay co pastoreo con bovinos. En este caso pueden surgir al menos 2 problemáticas: comenzar a tener problemas reproductivos en los ovinos del predio causados por BVDV, y también el ovino podría oficiar de posible eventual “reservorio” del virus para el bovino. Por tanto, entendemos que sería muy útil e interesante analizar muestras de estas 2 especies de animales.

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