Tesis de Doctorado en Ciencias Biológicas
PEDECIBA

Caracterización molecular de virus gastroentéricos bovinos en muestras clínicas y ambientales de rodeos de carne y leche del Uruguay

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"Si cruza los puentes de la capital, verá que esperando, verá que esperando hay medio Uruguay"

"La tropeada" - Larbanois-Carrero

A mis padres.
A Macarena.
A mis hijos.

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Resumen

En Uruguay, las exportaciones de carne bovina y productos lácteos se encuentran entre los principales rubros económicos. Por otro lado, las enfermedades que afectan a los terneros en las primeras semanas de vida son una importante causa de pérdidas económicas. Las principales causas de muerte en terneros de hasta dos meses de edad reportadas a nivel mundial son las infecciones que causan diarrea neonatal y las neumonías, siendo ambos síndromes de etiología múltiple. En el caso de la diarrea, puede ser provocada, entre otras causas, por rotavirus bovino del grupo A (RVA), coronavirus bovino (BCoV), norovirus bovino (BoNoV), torovirus bovino (BToV) y los astrovirus bovino (BoAstV), aunque para este último, el rol aún no está definido.

De lo antepuesto se desprende la importancia y necesidad de conocer los virus que afectan la producción ganadera tanto de carne como de leche en nuestro país, y por eso esta tesis tuvo como objetivo determinar la frecuencia de detección y caracterizar molecularmente RVA, BCoV, BoNoV, BToV y BoAstV en muestras clínicas y ambientales de rodeos de carne y leche del Uruguay.

A nivel ambiental se analizaron 202 muestras de agua utilizadas para el consumo de los terneros en tambos, utilizando a RVA como indicador de contaminación viral. El virus fue detectado mediante transcripción reversa seguida de reacción en cadena de la polimerasa en tiempo real (RT-qPCR) en el 35,1% de las muestras analizadas, con un marcado patrón de estacionalidad con mayor frecuencia de detección en los meses fríos por sobre los meses cálidos, y se confirmó la viabilidad de las partículas virales detectadas mediante aislamiento en cultivos celulares.

A nivel clínico se analizaron muestras de materia fecal y contenido intestinal de terneros provenientes de predios ganaderos dedicados a la producción de carne o leche.

En el caso de RVA fue detectado en el 57% de las 833 muestras analizadas para este virus mediante RT-qPCR, con significativamente mayor frecuencia de detección en terneros de tambos (60%) que en terneros de establecimientos dedicados a la producción de carne (28%, $p<0,001$). Este virus fue detectado mayormente en casos con diarrea (72%) que sin diarrea (60%, $p<0,005$), siendo la edad de los terneros con diarrea por rotavirus significativamente menor a la de los terneros que presentaron infección asintomática ($p<0,05$). Además, la carga viral fue significativamente mayor en terneros con diarrea que en terneros sin diarrea ($p<0,01$). Por otro lado, la frecuencia de detección de RVA fue similar en terneros nacidos de madres vacunadas (vacunación dirigida a proteger a los teneros mediante la transferencia de inmunidad pasiva) como de madres no vacunadas, así como tampoco se observó ningún patrón de estacionalidad como ocurrió en las muestras ambientales. En cuanto a la variabilidad genética, se determinaron los G-tipos y P-tipos, observando una

gran variabilidad a nivel de ambos genes, pudiéndose determinar la combinación de G y P tipos en 57 cepas, detectando G6P[5], G6P[11], G10P[11], y G24P[33]. En este mismo sentido, se profundizó en la caracterización de algunas cepas a través de la constelación genómica de las mismas (considerando las combinaciones detectadas a la hora de seleccionarlas, pero con especial hincapié e interés en la cepa rara G24P[33]), obteniendo secuencias también de los genes VP6, NSP1, NSP2, NSP3, NSP4 y NSP5, obteniendo resultados sumamente interesantes en cuanto a los genotipos detectados. Los análisis filogenéticos también determinaron que la variabilidad genética de las cepas circulantes en los terneros en nuestro país es importante.

El BCoV fue detectado en el 7,8% de las 824 muestras analizadas para este virus. No se observaron diferencias estadísticamente significativas entre la frecuencia de detección en ganado de leche y de carne, ni entre terneros vivos y muertos. Otro factor analizado fue la diarrea, donde tampoco se observó diferencias significativas entre la presencia del virus en muestras con y sin diarrea. Por otro lado, sí se observó mayor frecuencia de detección del virus en muestras de terneros nacidos de madres vacunadas (vacunación dirigida a proteger a los teneros mediante la transferencia de anticuerpos pasivos maternos) que en muestras de terneros nacidos de madres no vacunadas y mayor frecuencia de detección del virus en meses fríos por sobre meses más cálidos; en ambos casos las diferencias fueron significativas. En cuanto a la variabilidad genética, si bien existe un único serotipo, se pudo observar que en el ganado uruguayo circulan dos linajes distintos, los cuales fueron analizados evolutivamente, donde se pudo determinar que uno provino de Argentina y el otro de Brasil. Por último, se analizó el uso de codones de BCoV, determinando que existe un sesgo en su uso, principalmente debido al sesgo mutacional, donde los codones terminados en U son mayormente utilizados, el contenido G+C influencia el uso de codones en los genes de BCoV, así como también la composición de dinucleótidos. Además, se observaron diferencias significativas en la preferencia de codones utilizados por el virus y los utilizados por los genes del hospedero (*Bos taurus*).

En cuanto a BoNoV, fue detectado en el 66,1% de las 761 muestras analizadas para este virus, siendo el virus más frecuententemente detectado en las muestras de materia fecal de terneros analizadas. La circulación del virus fue significativamente mayor en muestras de terneros de tambos que de terneros provenientes de establecimientos dedicados a la producción de carne. No se observó estacionalidad, ni diferencia significativa entre la detección en terneros vivos y muertos, pero si una amplia distribución geográfica del virus. Los análisis filogenéticos confirmaron la presencia de los dos genotipos principales de norovirus bovinos GIII.1 y GIII.2.

Otro de los virus analizados, BToV, fue detectado en el 8,9% de las 325 muestras analizadas para este virus. Todas las muestras positivas fueron provenientes de terneros de tambos. Se observó mayor frecuencia de detección

en muestras provenientes de terneros muertos que vivos, aunque la diferencia no fue estadísticamente significativa. Aunque hasta el momento no se distinguen genotipos, se evidenció la circulación de dos linajes distintos, los cuales fueron detectados principalmente en Colonia, sin mostrar patrón de estacionalidad.

Por último, se evidenció la presencia de BoAstV en las muestras analizadas, aún cuando su rol en la diarrea neonatal del ternero se encuentra en discusión. El 26% de las 500 muestras analizadas para este virus fue positiva, observándose 3 especies virales distintas circulando en el ganado uruguayo, una de ellas sin asignación de especie, trabajo que fue realizado en el marco de esta tesis, caracterizándola como una nueva especie, resultando ser la detectada con mayor frecuencia. Por otro lado, cumplido uno de los objetivos que era estandarizar metodologías para detectar los virus en muestras de materia fecal y contenido intestinal, el mismo fue extendido a otro tipo de tejidos como cerebro. En este caso particular, se detectó por primera vez en todo el hemisferio sur un astrovirus asociado a encefalitis, para el cual se confirmó su asociación con la enfermedad y además fue caracterizado a nivel del genoma completo.

En suma, esta tesis brinda información fundamental sobre la epidemiología molecular de estos virus entéricos que afectan a los bovinos en nuestro país, sirven de sustento para continuar con esta línea de investigación en salud animal. Los hallazgos obtenidos destacan la importancia de mantener una vigilancia epidemiológica sobre la circulación de estos virus en los rodeos, lo que contribuirá a una mejora de la producción bovina en Uruguay, redundando en una mejora económica para el país.

Índice

"Caracterización molecular de virus entéricos bovinos en muestras clínicas y ambientales de rodeos de carne y leche del Uruguay"

Agradecimientos I

Resumen III

Índice VI

1. Introducción 1

1.1. Los bovinos en Uruguay 1

1.2. Síndrome de diarrea neonatal 3

1.3. Rotavirus A bovino 4

1.3.1. Descubrimiento 4

1.3.2. Enfermedad 5

1.3.3. Características generales 5

1.3.4. Clasificación 6

1.3.5. Transmisión 7

1.3.6. Prevención 7

1.3.7. RVA en la región 8

1.4. Coronavirus bovino 8

1.4.1. Descubrimiento 8

1.4.2. Enfermedad 9

1.4.3. Características generales 9

1.4.4. Clasificación 10

1.4.5. Transmisión 10

1.4.6. Prevención 10

1.4.7. BCoV en la región 10

1.5. Norovirus bovino 11

1.5.1. Descubrimiento 11

1.5.2. Enfermedad 11

1.5.3. Características generales 11

1.5.4. Clasificación 12

1.5.5. Transmisión 12

1.5.6. Prevención 13

1.5.7. BoNoV en la región 13

1.6. Torovirus bovino 13

1.6.1. Descubrimiento 13

1.6.2. Enfermedad 13

1.6.3. Características generales 14

1.6.4. Clasificación 15

1.6.5. Transmisión 15

1.6.6. Prevención 15

1.6.7. BToV en la región 15

1.7. Astrovirus bovino 15

1.7.1. Descubrimiento 15

1.7.2. Enfermedad 16

1.7.3. Características generales 16

1.7.4. Clasificación 16

1.7.5. Transmisión 17
1.7.6. Prevención 17
1.7.7. BoAstV en la región 17
1.8. Virus en el ambiente 17
1.9. Relevancia 19

2. Hipótesis 20

3. Objetivos 20
3.1. Objetivo general 20
3.2. Objetivos específicos 20

4. Materiales, métodos, resultados y discusión 21

A. Sección de estudios de virus a partir de muestras ambientales 22
A.1. Virus en el ambiente 22
A.2. Estudio de la contaminación viral en aguas utilizadas para el consumo de terneros en tambos de Uruguay 23
Artículo 1. Prevalencia y viabilidad de rotavirus del grupo A en fuentes de agua de tambos 23

B. Sección de estudios de virus a partir de muestras clínicas 34
B.1. Virus en terneros 34
B.2. Estudio de coronavirus bovino en muestras clínicas y análisis evolutivos 35
Artículo 2. Coronavirus bovino en Uruguay: diversidad genética, factores de riesgo e introducciones transfronterizas desde países vecinos 35
Artículo 3. Análisis del sesgo de uso de codones en todo el genoma de coronavirus bovino 46
B.3. Estudio de rotavirus en muestras clínicas 54
Artículo 4. Análisis filogenéticos de RVA bovino en Uruguay revelan la circulación de genotipos comunes y no comunes, y sugieren transmisión interespecie 54
B.4. Estudio de astrovirus bovino en muestras clínicas 78
Artículo 5. Vigilancia de astrovirus bovino en Uruguay revela una alta tasa de detección de una nueva especie de *Mamastrovirus* 78
Artículo 6. El primer caso de encefalitis asociada a astrovirus bovino en el hemisferio sur (Uruguay), revela evidencia de introducción viral a las Américas desde Europa 89
B.5. Estudio de norovirus en muestras clínicas 98
Artículo 7. Detección, factores de riesgo y diversidad molecular de norovirus GIII en terneros en Uruguay 98
Artículo 8. Historia evolutiva y dinámica espacio-temporal de norovirus GIII: desde la emergencia hasta la clasificación en 4 genotipos 108
B.6. Estudio de torovirus bovino en muestras clínicas 118
Artículo 9. Primer reporte y caracterización genética de torovirus bovino en Uruguay . 118
Artículo 10. Frecuencia de detección y factores de riesgo asociados a la infección por torovirus bovino en Uruguay 133
B.7. Anexo 139
Artículo 11. Causas de diarrea neonatal de terneros y mortalidad en tambos a base de pastura en Uruguay: un estudio de casos y controles 139
Artículo 12. Efecto de la administración de cepas de *Lactobacillus spp.* sobre la diarrea neonatal, parámetros inmunológicos y abundancia de patógenos en terneros pre-destete. 140
Artículo 13. Enfermedades asociadas con el virus de la diarrea viral bovina subtipos 1a y 2b en ganado de carne y leche en Uruguay 141

Artículo 14. Encefalitis asociada a astrovirus bovino neurotrópico, ¿una enfermedad subdiagnosticada en Sudamérica? 142
Artículo 15. Desarrollo de un producto basado en IgY para prevenir la diarrea por coronavirus bovino 144

5. Discusión general 145

6. Conclusiones 150

7. Perspectivas 151

8. Referencias bibliográficas 152

9. Artículos no relacionados a la tesis 160

Artículo 16. La historia evolutiva y dinámica espacio-temporal del linaje NC del virus de la tristeza de los cítricos 160
Artículo 17. Un análisis exhaustivo de la composición del genoma y los patrones de uso de codones de coronavirus emergentes 161
Artículo 18. Evidencia de creciente diversificación de cepas emergentes de SARS-CoV-2 162
Artículo 19. Detección molecular de norovirus en muestras de aguas residuales uruguayas revela una alta diversidad genética y el reemplazo de variantes GII.4 a lo largo del tiempo 163
Artículo 20. Bocavirus humano: detección, cuantificación y caracterización molecular en aguas residuales y superficiales en Uruguay 164
Artículo 21. Un extenso estudio de campo revela la circulación de nuevas variantes genéticas del subtipo 1a de BVDV en Uruguay 165
Artículo 22. Evaluación de la contaminación bacteriana como indicador de contaminación viral en un acuífero sedimentario en Uruguay 166
Artículo 23. Modelado del transporte de rotavirus y norovirus humanos en sistemas de matrices acuáticas estandarizados y en suelo natural 167

10. Consideraciones finales 168

1. Introducción

1.1. Los bovinos en Uruguay

En Uruguay, según datos oficiales del Ministerio de Ganadería Agricultura y Pesca (MGAP) – Estadísticas Agropecuarias (DIEA) a través de la Declaración Jurada ante la División Contralor de Semovientes (DICOSE), en el año 2018 había un total de 11.468.000 vacunos, de los cuales 2.799.000 (24,4%) eran terneros/as. Además, se reportó un ingreso total por exportaciones de 7.495 millones de dólares, de los cuales el 74% (5.547 millones de dólares) está vinculado al sector agropecuario, y más precisamente, el 37% del total de las exportaciones de nuestro país está relacionado a los bovinos, totalizando 2.834 millones de dólares, siendo la exportación de carne bovina el principal producto exportado (22,3%), mientras que los productos lácteos (9,0%) y los bovinos en pie (3,6%) se ubicaron también dentro de los 5 principales productos de exportación. La producción de carne en ese mismo año fue de 1.100 millones de kilogramos y de leche comercial fue de 2.173 millones de litros.

En el plano internacional, Uruguay se ubica entre los principales países exportadores de carne vacuna, así como también de leche y derivados (FAO, 2019; IDR, 2013). Además, en Uruguay el consumo *per cápita* de carne vacuna es el más alto a nivel mundial. Por otro lado, en 5% del territorio del país, se produce leche para alimentar anualmente a más de 20 millones de personas (INALE, 2019).

En el plano local, el consumo de carne como fue mencionado anteriormente es muy importante en nuestro país, siendo base fundamental de la alimentación de los uruguayos. Según datos oficiales aportados por INAC y Presidencia en junio de 2019, en el 98% de los hogares se consume carne vacuna, mientras que el 99% de los uruguayos consume carne vacuna. Además, 9 de cada 10 hogares no tienen miembros que no consuman carne vacuna, 3 de cada 4 uruguayos consume platos con carne vacuna en promedio diario, 8 de cada 10 uruguayos consume carne vacuna al menos 2 días a la semana, y 4 de cada 10 lo hace al menos 4 días por semana. La carne vacuna en más de la mitad de los almuerzos diarios y en casi la mitad de las cenas diarias (INAC, 2019). Además, debe añadirse el consumo de carne en otras formas, como los chacinados. Por otra parte, el consumo de productos lácteos y derivados, es también muy importante en nuestro país. El consumo anual de leche es de 230 litros *per cápita*, más del doble del consumo mundial promedio (INALE, 2019). A eso debe sumarse también el consumo de yogur, quesos y otros derivados como la manteca.

Figura 1. Ternero amamantándose. Los terneros en los establecimientos ganaderos para producción de carne son, en general, criados al pie de la madre (crianza extensiva).

Figura 2. Terneros en un tambo. Los terneros en los tambos son, en general, criados artificialmente en guacheras (crianza intensiva).

Figura 3. Crianza intensiva de terneros para producción de carne.

1.2. Síndrome de diarrea neonatal

El estado de salud de los terneros en los primeros días de vida es fundamental para su supervivencia y posterior desarrollo, por lo que las enfermedades que afectan a los terneros en esos días son una importante causa de pérdidas económicas a nivel mundial (Waltner-Toews *et al*. 1986; Donovan *et al*., 1998; Windeyer *et al*. 2014). Las principales causas de muerte en terneros de hasta dos meses de edad reportadas a nivel mundial son las infecciones que causan diarrea neonatal (DNT) y las neumonías (Windeyer *et al*., 2014).

La DNT es la principal causa de muerte de terneros recién nacidos (Hur *et al*., 2013; Hötzel *et al*., 2014; Urie *et al*., 2018), y tiene un impacto negativo en el bienestar animal, generando pérdidas económicas para la industria ganadera (Waltner-Toews *et al*. 1986; Donovan *et al*. 1998; Windeyer *et al*. 2014). La DNT es un problema importante a nivel mundial, pero particularmente donde hay una intensificación de la ganadería. Sin embargo, se pueden producir pérdidas severas esporádicamente en establecimientos con ganadería extensiva, por lo que no se debe subestimar la importancia para el pequeño productor (Selman, 1981).

La DNT afecta principalmente a terneros recién nacidos debido a que éstos nacen desprovistos de anticuerpos que le confieran protección frente a los patógenos y dependen totalmente de adquirirlos a través de la ingesta del calostro, por lo que los terneros deben recibir cantidades significativas del calostro de su madre tan pronto como sea posible después del nacimiento, y preferentemente dentro de las primeras 6 horas de vida, donde el intestino del ternero tiene la permeabilidad adecuada para absorver inespecíficamente macromoléculas hacia la circulación general. Si absorben suficientes inmunoglobulinas, probablemente se volverán más resistentes a los efectos de la DNT (Selman, 1981).

Aunque la industria ganadera ha realizado grandes mejoras con el manejo del rebaño, las instalaciones y el cuidado de los animales, la alimentación y la nutrición, y el uso oportuno de productos biofarmacéuticos, la DNT sigue siendo problemática debido a la naturaleza multifactorial de la enfermedad. La prevención y el control de la DNT deben basarse en una buena comprensión de las complejidades de la enfermedad, tales como múltiples patógenos, coinfección, factores ambientales y nutricionales, de manejo durante el período de parto antes de los brotes de la enfermedad, y durante la crianza, especialmente en los rodeos de tambo (Cho y Yoon, 2014).

En cuanto a los factores infecciosos, son múltiples los patógenos entéricos (virus, bacterias y protozoos) que suelen estar involucrados en el desarrollo de la enfermedad. La coinfección se observa con frecuencia en terneros diarreicos, aunque un solo patógeno primario puede ser la causa en algunos casos. La prevalencia de cada incidencia de patógenos y enfermedades puede variar según la ubicación geográfica, las prácticas de manejo y el tamaño

del rebaño (Cho y Yoon, 2014). Los patógenos más prevalentes suelen ser: rotavirus bovino grupo A (RVA), coronavirus bovino (BCoV), *Escherichia coli* y *Cryptosporidium parvum*. Otros agentes virales que han sido también asociados a la DNT son: norovirus bovino (BoNoV), torovirus bovino (BToV) y los astrovirus bovino (BoAstV), aunque para este último el rol aún no está definido.

La causa de un brote de DNT rara vez se busca, y mucho menos se logra identificar, incluso en países desarrollados con todos los beneficios de una buena comunicación y servicios veterinarios bien organizados. La DNT es tan común en ciertas circunstancias que los productores están acostumbrados a ver terneros diarreicos y buscan asesoramiento veterinario solo cuando el problema alcanza lo que consideran un nivel inaceptable. Otro problema para el diagnóstico es la presentación de terneros muertos o moribundos que ya han recibido alguna forma de terapia, principalmente con antibióticos. Sin embargo, la razón más importante para un diagnóstico deficiente es que se sabe poco sobre la prevalencia, la importancia relativa, las posibles interrelaciones y los efectos patogénicos de la gran cantidad de microorganismos que se ha demostrado, o se afirma, que son causales (Selman, 1981).

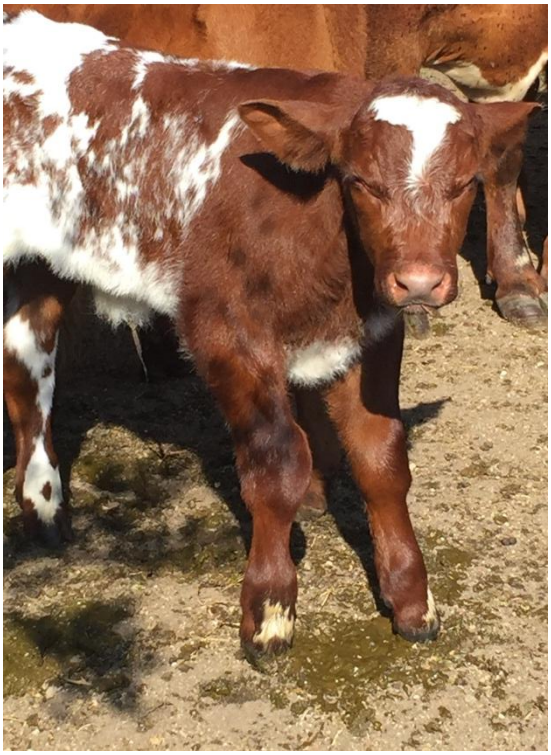


Figura 4. Ternero parado sobre materia fecal.

1.3. Rotavirus A bovino

1.3.1. Descubrimiento

En el año 1969, se confirmó por primera vez que un "virus tipo reo" era causante de diarrea en terneros (Mebus *et al*., 1969) y años más tarde fue nombrado rotavirus debido a que por microscopía electrónica tenían apariencia similar a una rueda (*Rota* en latín). Desde su descubrimiento, ha sido considerado como el principal agente causal de DNT (Garaicoechea *et al*., 2006), infligiendo graves pérdidas al sector ganadero (Dhama *et al*., 2009).

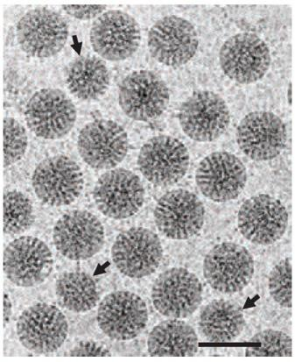


Figura 5. Micrografía electrónica de partículas de rotavirus. Se observan partículas de rotavirus con triple capa y algunas con doble capa (flechas) visualizadas por microscopía electrónica de transmisión. Barra, 100 nm. Tomado de Estes y Greenberg, 2013.

1.3.2. Enfermedad

La enfermedad generalmente se observa en terneros jóvenes de 2-8 semanas de edad y la susceptibilidad disminuye a medida que la edad avanza. En neonatos, la infección tiene un muy corto período de incubación, y se manifiesta con diarrea profusa y deshidratación severa. La diarrea se produce principalmente debido a la disminución en la eficacia de absorción de los enterocitos por causa de la infección con el virus y la gravedad puede variar desde una condición asintomática o subclínica a una enteritis severa. Además, la infección concurrente con patógenos secundarios puede aumentar la gravedad de la enfermedad (Dhama *et al*., 2009).

1.3.3. Características generales

RVA bovino al igual que el resto de los rotavirus del grupo A, son miembros del género *Rotavirus* dentro de la familia *Reoviridae*. Comparten características estructurales, genómicas y replicativas. Algunas de las características estructurales: son partículas icosaédricas de 100 nanómetros (nm) de diámetro (incluyendo las espigas), tienen una cápside con tres capas, son virus desnudos y dentro de la cápside están todas las enzimas para la producción de ARN mensajero (ARNm). Las características genómicas compartidas son: constan de 11 segmentos de ARN doble hebra (ARNdh), los segmentos de ARN purificados no son infecciosos, cada segmento de ARN codifica para al menos una proteína y los segmentos de ARN de diferentes virus se reordenan genéticamente con una alta frecuencia durante la co-infección de una célula. Por último, las características replicativas son: el cultivo

es facilitado por proteasas como tripsina o pancreatina, la replicación se da en el citoplasma, forman cuerpos de inclusión, la morfogénesis implica partículas envueltas transitorias, el nivel de calcio intracelular es importante para el ensamblado y la estabilidad del virus y las partículas virales son liberadas mediante lisis celular o por transporte vesicular no clásico en células epiteliales polarizadas (Estes y Greenberg, 2013).

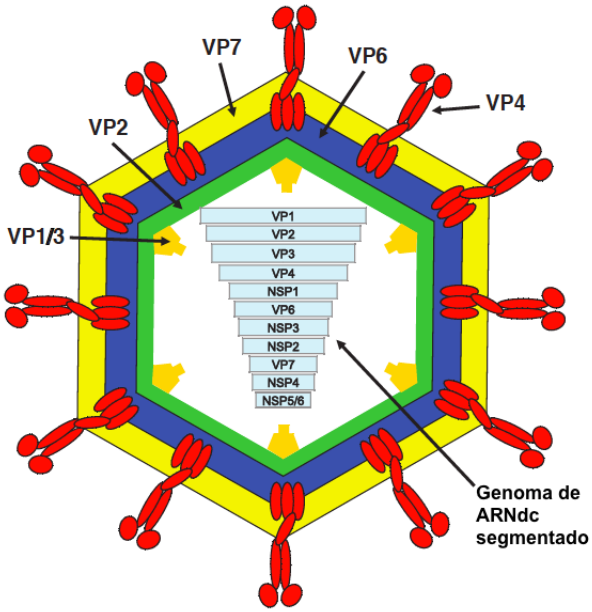


Figura 6. Diagrama esquemático de las partículas de rotavirus. La partícula está compuesta por tres capas proteicas concéntricas (VP7, VP6 y VP2) y la proteína VP4 que atraviesa las capas VP6 y VP7 y se extiende hacia afuera de la partícula. Un complejo de transcripción de VP1 y VP3 está dentro de la capa VP2. El genoma de ARN doble hebra viral es segmentado. Tomado y modificado de Estes y Greenberg, 2013.

1.3.4. Clasificación

Los rotavirus (RV) se clasifican serológicamente en 10 grupos distintos, llamados RVA, RVB, RVC, RVD, RVE, RVF, RVG, RVH, RVI, y RVJ (https://talk.ictvonline.org/taxonomy/). Taxonómicamente, los RVA, según el Comité Internacional para la Taxonomía de los Virus (ICTV) se clasifican actualmente en: *Riboviria* › *Orthornavirae* › *Duplornaviricota* › *Resentoviricetes* › *Reovirales* › *Reoviridae* › *Sedoreovirinae* › *Rotavirus* › *Rotavirus A.*

Los RVA, además, se clasifican antigénicamente en serotipos (basados en epítopos neutralizantes de las proteínas virales VP4 y VP7), y genéticamente en genotipos, con ciertas diferencias entre la clasificación de serotipos y genotipos en VP4 (Estes y Greenberg, 2013). Hasta el momento hay 35 genotipos VP4 ([P]1-35) y 27 genotipos VP7 (G1-27) dentro del grupo RVA (Matthijnssens *et al.*, 2011), de los cuales 11 P tipos (P[1], P[3], P[5], P[6], P[7], P[11], P[14], P[17], P[21], P[29] y P[33]) y 12 G tipos (G1, G2, G3, G5, G6, G8, G10, G11, G15 G17, G21 y G24) han sido identificados en bovinos. Sin embargo, los genotipos G6,

G8 y G10 asociados a P[1], P[5] y P[11] son los más comúnmente encontradas en los bovinos (Papp *et al*., 2013).

La clasificación dentro de RVA, se realiza comúnmente mediante un sistema binario con los distintos tipos de VP4 y VP7, siendo GXP[Y], donde X es el G tipo e Y es el P tipo. La falta de suero o anticuerpos monoclonales para diferentes tipos de VP4 disponibles ha obstaculizado la clasificación de VP4 en serotipos. Los genotipos de VP4 y VP7 son determinados por análisis de secuencia, mientras que los serotipos son determinados por la reactividad de las cepas individuales o recombinantes seleccionadas con antisueros policlonales o monoclonales. Para VP7, se ha establecido una correlación entre el genotipo y el serotipo. Esta correlación es mucho menos clara para VP4 aunque una región variable, VP8*, que se extiende del aminoácido (aa) 71 al 204 de VP4 puede definir epítopos específicos de tipo P (Estes y Greenberg, 2013). De todas formas existe una clasificación más amplia basada en la secuencia nucleotídica de cada segmento, en la cual se le asigna a cada uno de los 11 segmentos VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 un genotipo particular utilizando las abreviaciones Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (donde x corresponde a números partiendo del 1), respectivamente (Matthijnssens *et al*., 2008).

1.3.5. Transmisión

La transmisión es generalmente por la ruta fecal-oral y son muy contagiosos; basta solamente una dosis infecciosa de cultivo celular para causar la enfermedad en un hospedero completamente susceptible. Además, son muy estables en el ambiente y son excretados en grandes cantidades en la materia fecal, aumentando aún más la posibilidad de transmisión (Estes y Greenberg, 2013).

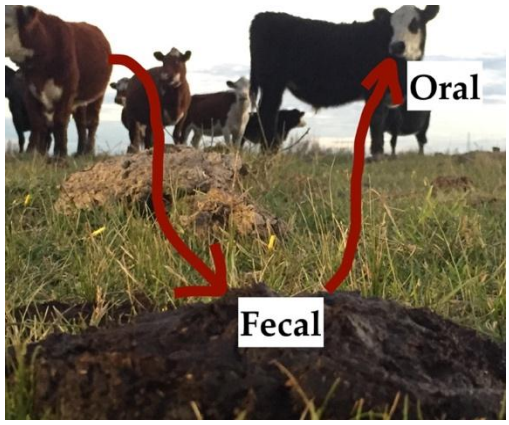


Figura 7. Transmisión fecal-oral.

1.3.6. Prevención

La estrategia actual para controlar la enfermedad en el ganado se basa en la vacunación de las vacas durante el último tercio de gestación, para proteger a

los terneros por transferencia de anticuerpos pasivos maternos a través de la ingesta de calostro. Varias vacunas inactivadas están disponibles en el mercado y la vacunación sistemática mostró ser eficaz para reducir la morbilidad por diarrea, al menos en los países vecinos. Sin embargo, estas vacunas comerciales se preparan con el sobrenadante de cultivos celulares infectados con cepas de referencia que pueden no representar las cepas de RVA que circulan en nuestro país. Estudios filogenéticos de los RVA circulantes en el ganado, contribuyen a una mejor comprensión de la epidemiología de este patógeno, que se traduce en información importante para evaluar la necesidad de actualizar las cepas vacunales, y añadir datos completos para dilucidar los mecanismos de evolución del virus (Badaracco *et al*., 2013).

1.3.7. RVA en la región

Estudios previos en la región demuestran que RVA se encuentra ampliamente distribuido, causando más del 60% de los casos de brotes de diarrea en rodeos de carne y leche argentinos (Garaicoechea *et al*., 2006). En Argentina, las cepas de RVA prevalentes que circulan en el ganado pertenecen a los genotipos G6, G10, P[5] y P[11]. Además, observaron una distribución diferencial clara de los tipos G y P dependiendo del tipo de explotación (carne o leche) (Garaicoechea *et al*., 2006; Badaracco *et al*., 2012, 2013; Bertoni *et al*., 2020). Para nuestro país no se conocen cuáles son los genotipos que circulan actualmente y las cepas utilizadas en las vacunas son en base a datos de la región, lo cual puede no reflejar la realidad, reduciendo la efectividad de la vacunación.

1.4. Coronavirus bovino

1.4.1. Descubrimiento

El primer reporte de BCoV fue en el año 1971, durante un ensayo de prueba de una vacuna oral contra rotavirus; en las heces de un ternero negativo para rotavirus se encontraron partículas tipo coronavirus (Stair *et al*., 1972; Mebus *et al*., 1973).

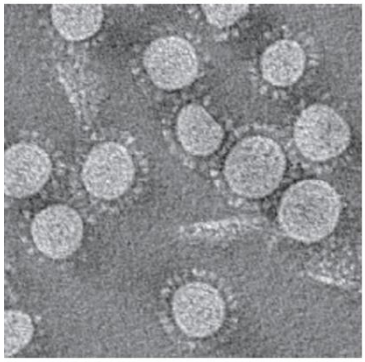


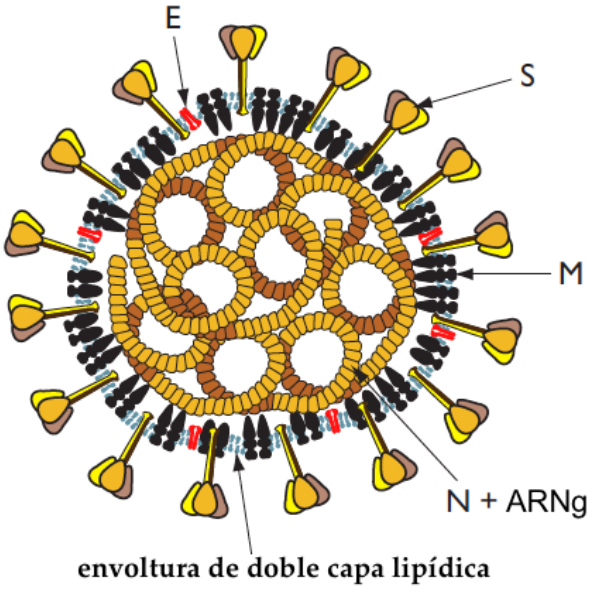
Figura 8. Micrografía electrónica de partículas de coronavirus. Tomada de Masters y Perlman, 2013.

1.4.2. Enfermedad

Actualmente, está ampliamente reconocido como uno de los principales agentes causales de diarreas neonatales en terneros. Además, es considerado el segundo mayor patógeno causante de muertes en terneros, demostrando la gran severidad de la enfermedad causada (Blanchard, 2012). Además de diarrea neonatal, puede causar infecciones respiratorias y disentería de invierno en bovinos adultos (Blanchard, 2012; Saif, 2010). El virus se multiplica en las células de las criptas intestinales, disminuyendo la capacidad digestiva y de absorción conduciendo a la diarrea, con pérdida de agua y electrolitos. En infecciones graves, la diarrea puede causar deshidratación, acidosis e hipoglucemia, y la muerte puede ocurrir debido a un shock agudo e insuficiencia cardíaca. La gravedad de la enteritis varía tanto con la edad y el estado inmunológico del ternero así como también por la dosis infecciosa y la cepa del virus, desarrollando diarrea más rápidamente y más severa en terneros menores de tres meses, afectando típicamente a terneros de entre una y dos semanas de edad (Clark, 1993).

1.4.3. Características generales

Los BCoV son virus envueltos de forma pleiomórfica con un diámetro de entre 65 y 210 nm. Están cubiertos por una doble capa de proyecciones de superficie cortas (hemaglutinina esterasa, HE) y largas (espiga, S), las cuales están implicadas en la unión a los receptores celulares por lo que son importantes para la inmunidad y las vacunas (Saif, 2010). Las otras dos proteínas estructurales importantes son la proteína de la nucleocápside (N) y la glicoproteína integral de membrana (M). El genoma consiste de ARN de simple cadena de polaridad positiva no segmentado con un tamaño de 27-30 kilobases (kb) y está organizado en siete regiones las cuales contienen uno o más marcos abiertos de lectura (ORF). Estas regiones están separadas por sitios de reiniciación que contienen la señal para la transcripción de los ARN mensajeros subgenómicos. Hacia el extremo 5′ del genoma se codifican las proteínas no estructurales incluyendo la ARN polimerasa viral, mientras que el orden de las proteínas estructurales es 5′-HE-S-M-N-3′ (Clark, 1993). Al igual que los ARNm



celulares tiene cap en el extremo 5' y cola poli A en el extremo 3' (Masters, 2006).

Figura 9. Diagrama esquemático de las partículas de coronavirus. Se muestran las principales proteínas estructurales del virión: S, M, E, y N. Se muestra también el ARN genómico y la bicapa lipídica. Tomada de Masters y Perlman, 2013.

1.4.4. Clasificación

Los BCoV son miembros de la familia *Coronaviridae* dentro del orden *Nidovirales*; particularmente pertenecen a la subfamilia *Coronavirinae* y al género *Betacoronavirus* (también llamado grupo 2 o grupo II) (Masters y Perlman, 2013). Recientemente, el ICTV, reorganizó los coronavirus y especies que anteriormente se consideraban distintas, hoy en día son la misma. Este es el caso de la especie *Betacoronavirus 1*, la cual contiene virus que afectan distintas especies animales y humanas; algunos miembros de esta especie son el coronavirus humano OC43, el coronavirus equino, el virus de la encefalomielitis hemaglutinante porcina y el propio BCoV. Además, otro miembro importante del género *Betacoronavirus* es el coronavirus asociado al síndrome respiratorio severo agudo (SARS-CoV) (Masters y Perlman, 2013), y el recientemente descrito y causante de la pandemia por COVID-19, el SARS-CoV-2. Actualmente, la clasificación taxonómica para BCoV, según el ICTV es: *Riboviria › Orthornavirae › Pisuviricota › Pisoniviricetes › Nidovirales › Cornidovirineae › Coronaviridae › Orthocoronavirinae › Betacoronavirus › Embecovirus › Betacoronavirus 1*. Hasta el momento todos los aislados de BCoV pertenecen a un mismo serotipo, con variaciones antigénicas menores (Clark, 1993).

1.4.5. Transmisión

La transmisión puede ser tanto por vía fecal-oral como respiratoria y se da principalmente en los meses de invierno. Además, se ha descrito que los coronavirus son capaces de mantenerse estables e infecciosos durante semanas en diferentes tipos de matrices ambientales, incluida el agua (Casanova *et al*., 2009; Mullis *et al*., 2012).

1.4.6. Prevención

La estrategia actual para controlar la enfermedad en el ganado se basa en la vacunación de las vacas preñadas para proteger a los terneros por transferencia de anticuerpos pasivos maternos a través de la ingesta de calostro (Bok *et al*., 2018).

1.4.7. BCoV en la región

La prevalencia de BCoV en la región es muy variable, de 2-35% en muestras de diarrea de terneros (Corbett *et al*., 1989; Brandao *et al*., 2006; Stipp *et al*., 2009; Bok *et al*., 2015). Ha sido ampliamente estudiado en la región, debido a que es un patógeno que impacta negativamente en los países con producción

ganadera como son los países de Sudamérica. Sin embargo, no hay datos publicados sobre la prevalencia de BCoV en nuestro país.

1.5. Norovirus bovino

1.5.1. Descubrimiento

Los BoNoV fueron descritos por primera vez en el año 1978 junto con los astrovirus bovinos en muestras de heces de terneros diarreicos (Woode y Bridger, 1978). Si bien se encuentra estudiado en menor medida que rotavirus y coronavirus, diversos estudios confirman que los BoNoV están ampliamente presentes en casos de diarrea en el ganado de diferentes países, variando desde 3% a más de 30% (van der Poel *et al*., 2000; Deng *et al*., 2003; van der Poel *et al*., 2003; Milnes *et al*., 2007; Ferragut *et al*., 2016). Además, estudios serológicos indican que la circulación y exposición a este virus es muy alta, llegando a detectar anticuerpos contra BoNoV en más del 99% de las muestras estudiadas (Deng *et al*., 2003).

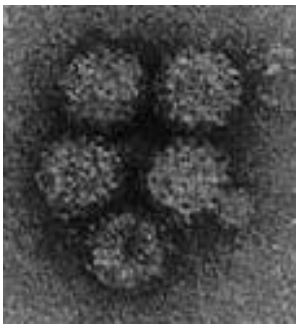


Figura 10. Micrografía electrónica de partículas de norovirus. Tomada de van der Poel *et al*., 2003.

1.5.2. Enfermedad

La enfermedad suele ser más severa en terneros de entre tres semanas y dos meses y los signos clínicos suelen ser enteritis no hemorrágica, diarrea leve, anorexia transitoria y mala absorción a nivel del intestino (Bridger *et al*., 1984; Scipioni *et al*., 2008). La patogenicidad de BoNoV se debe principalmente a lesiones en el intestino delgado, además de focos hemorrágicos y acortamiento de las vellosidades (Woode y Bridger, 1978).

1.5.3. Características generales

Los BoNoV son virus no envueltos de 27 a 35 nm de diámetro, con una cápside de simetría icosaédrica con 180 moléculas de la proteína de la cápside organizadas en 90 dímeros y cuya superficie muestra 32 depresiones en forma de copa y arcos que sobresalen (calici deriva de la palabra en latín *calyx*, que significa cáliz o copa) (Prasad *et al*., 1999; Scipioni *et al*., 2008). El genoma es de ARN simple hebra de polaridad positiva de aproximadamente 7,5 kb y contiene 3 ORF (Scipioni *et al*., 2008). El extremo 5′ se encuentra unido a una proteína

viral llamada VPg, no posee ni sitio de entrada al ribosoma ni cap y se supone que VPg interacciona con los componentes de la maquinaria traslacional iniciando la traducción del ARN viral (Daughenbaugh *et al*., 2003; Goodfellow *et al*., 2005). El extremo 3′ contiene una cola poli-A. El ORF 1 (localizado hacia el extremo 5′ del ARN) codifica para al menos 6 proteínas no estructurales (p48, NTPasa, p22, VPg, 3CLPro y ApAd), el ORF 2 para la VP1 y el ORF 3 para la VP2 (Scipioni *et al*., 2008).

Figura 11. Esquema del genoma de norovirus. Se muestran las principales proteínas codificadas, así como también la organización de los 3 ORF. Es de notar el solapamiento entre el ORF1 y el ORF2, región donde ocurre recombinación con mayor frecuencia.

1.5.4. Clasificación

Los BoNoV se clasifican dentro del género *Norovirus* de la familia *Caliciviridae*. La clasificación completa según el ICTV es: *Riboviria › Orthornavirae › Pisuviricota › Pisoniviricetes › Picornavirales › Caliciviridae › Norovirus*. Según el análisis de la secuencia de la cápside pertenecen al genogrupo III del género *Norovirus*. A su vez los BoNoV se clasifican en 2 genotipos del genogrupo III (GIII.1 y GIII.2) (Scipioni *et al*., 2008), aunque recientemente un posible nuevo genotipo fue descrito (Wang *et al*., 2019).

1.5.5. Transmisión

La transmisión es principalmente vía fecal-oral, aunque también se sugiere que otra vía natural de infección podría ser el tracto respiratorio a través de partículas de aerosol en vómito (Hall *et al*., 1984; Sawyer *et al*., 1988; Scipioni *et al*., 2008). Los calicivirus se caracterizan por su gran estabilidad en el medio ambiente y la resistencia a la inactivación (Duizer *et al*., 2004; Rzezutka y Cook, 2004; Nims y Plasvic, 2013). Además, bajas dosis infecciosas así como también su gran diversidad de cepas aumentan el riesgo de infección (Scipioni *et al*., 2008).

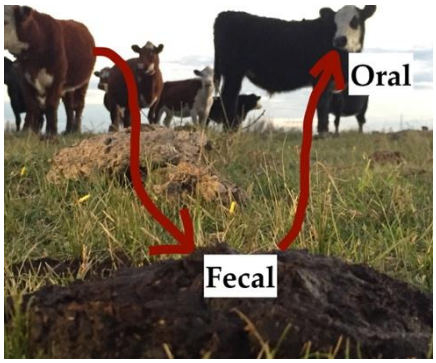


Figura 12. Transmisión fecal-oral.

1.5.6. Prevención

No hay vacuna comercial disponible para BoNoV, por lo que no hay, actualmente, una estrategia de prevención dirigida especialmente a este virus.

1.5.7. BoNoV en la región

En lo que refiere a BoNoV, la información en la región es muy escasa, contando únicamente con un estudio de Argentina, donde se determinó una frecuencia de detección de 3,3%. Además, una de las cepas caracterizadas, demostró ser recombinante, con un genotipo posiblemente nuevo (Ferragut *et al*., 2016). Para Uruguay no hay trabajos publicados sobre su prevalencia, ni mucho menos se conocen los genotipos que circulan, por lo que no se toman medidas preventivas contra este virus.

1.6. Torovirus bovino

1.6.1. Descubrimiento

Los BoToV fueron detectados por primera vez en el año 1979 (Woode *et al*., 1982) en un establecimiento cercano a la ciudad Breda del estado Iowa, Estados Unidos; de ahí que inicialmente se denominaron Breda virus. El nombre torovirus proviene del Latín *torus*, debido a que la forma de la nucleocápside es tubular alargada con simetría helicoidal y puede doblarse confiriéndole la forma de un disco bicóncavo o con forma de riñón; las partículas virales poseen una envoltura con peplómeros la cual contiene esta nucleocápside (Horzinek *et al*., 1987).

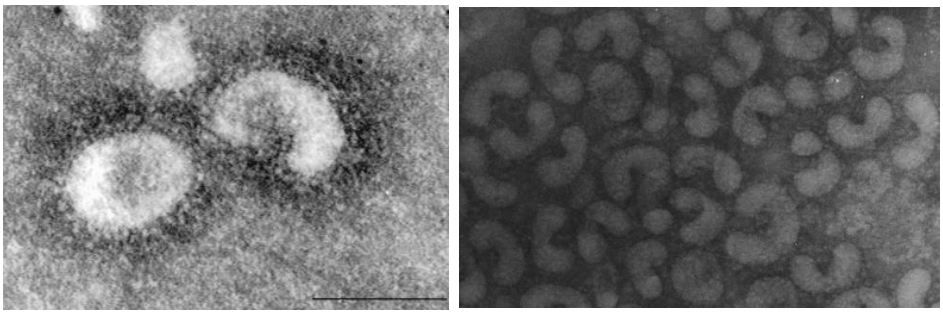


Figura 13. Micrografías electrónicas de partículas de torovirus. Se pueden observar partículas de diferentes formas (pleomórficas) incluyendo algunas en forma de toro y media luna. Barra, 100 nm. Tomadas de Duckmanton *et al*., 1998 y Hoet y Saif, 2004.

1.6.2. Enfermedad

En cuanto a la enfermedad, los BoToV se han descrito como patógenos entéricos que causan diarrea leve a profusa en terneros en sus primeros meses de vida (Hoet y Saif, 2004), llegando a generar riesgo de vida en estos animales (Horzinek *et al*., 1987). Además, pueden afectar al ganado adulto con signos clínicos similares a la disentería de invierno producida por los coronavirus;

éstos signos son diarrea acuosa y anorexia (que traen aparejado pérdida de peso), y en el caso de las vacas lecheras se le suma disminución en la producción de leche (Aita *et al*., 2012). Al igual que los coronavirus, pueden afectar también el tracto respiratorio causando importantes pérdidas (Aita *et al*., 2012; Hoet *el al*., 2002). La patogenicidad se debe principalmente a la enteritis. A nivel macroscópico puede observarse delgadez en la pared intestinal, mientras que histológicamente se observa fusión y atrofia de las vellosidades y descamación epitelial desde el yeyuno medio hasta el intestino delgado inferior, además de áreas de necrosis en el intestino grueso (Koopmans y Horzinek, 1994). Se aprecia infección y necrosis de las vellosidades y de los enterocitos de la cripta en el intestino delgado (Woode *et al*., 1982, Woode, 1984) que parecen únicas para BoToV; otros virus infectan células de las vellosidades pero no las células de la cripta, o viceversa (Hall, 1987).

1.6.3. Características generales

El genoma está compuesto de una molécula de ARN simple hebra de polaridad positiva y tiene un largo de 28.475 nucleótidos (Draker *et al*., 2006). El mismo contiene dos ORFs superpuestos, ORF1a ORF1b, que codifican las proteínas implicadas en la replicación, como por ejemplo una serín proteasa, una cisteín proteasa, un dominio ADRP (único para la familia *Coronaviridae*), varios dominios hidrófobos que son típicos de una replicasa de los nidovirus, dominios de polimerasa y helicasa (en el ORF1b), así como secuencias implicadas en el desplazamiento de marcos ribosómicos, incluyendo la secuencia resbaladiza conservada UUUAAAC y dos estructuras potenciales pseudoknot (Draker *et al*., 2006). Hacia el extremo 3´se encuentran codificadas las proteínas estructurales S (peplómeros), M (envoltura), HE (hemaglutinin-esterasa) y N (nucleocápside) (Draker *et al*., 2006; Duckmanton *et al*., 1998). En ambos extremos (5′ y 3′), flanqueando la región codificante, el genoma contiene dos regiones no codificantes (Draker *et al*., 2006).

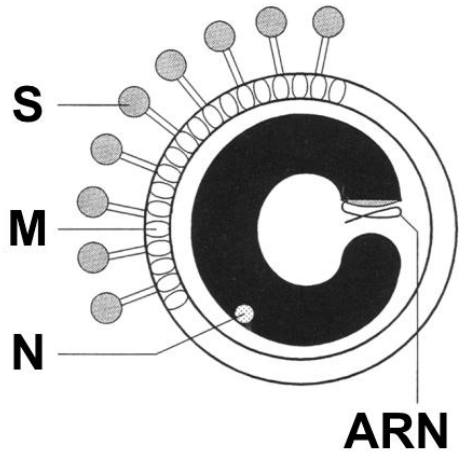


Figura 14. Diagrama esquemático de las partículas de torovirus. Se muestran las principales proteínas estructurales del virión: S, M, y N, y el genoma de ARN. Modificado de Snijder y Horzinek, 1993.

1.6.4. Clasificación

Inicialmente se denominaron Breda virus y se agruparon en una nueva familia denominada *Toroviridae* de acuerdo a la morfología y la estructura del virión (Horzinek *et al*., 1987). En 1992 los torovirus fueron reclasificados como un nuevo género dentro de la familia *Coronaviridae* (Cavanagh *et al*., 1993), pero luego en el año 2003, esta familia se dividió en dos subfamilias: *Coronavirinae* y *Torovirinae* (Gonzalez *et al*., 2003). Luego, los BoToV fueron reclasificados nuevamente pasando a formar parte del género *Torovirus* de la subfamilia *Torovirinae*, dentro de la familia *Coronaviridae*. Sin embargo, la clasificación actual según el ICTV es: *Riboviria › Orthornavirae › Pisuviricota › Pisoniviricetes › Nidovirales › Tornidovirineae › Tobaniviridae › Torovirinae › Torovirus › Renitovirus › Bovine torovirus*.

1.6.5. Transmisión

La transmisión es principalmente mediante la vía fecal-oral aunque también pueden ser transmitidos por vía respiratoria (Holmes, 2001).

1.6.6. Prevención

No hay vacuna comercial disponible para BToV, por lo que no hay, actualmente, una estrategia de prevención dirigida especialmente a este virus.

1.6.7. BToV en la región

Los datos publicados sobre BToV en la región son realmente escasos, contando únicamente con un reporte sobre la detección de este virus en Brasil, donde se observó una frecuencia de 6,2% (Nogueira *et al*., 2013). En Uruguay, no hay registros publicados sobre la presencia de este virus ni se toman medidas preventivas contra el mismo.

1.7. Astrovirus bovino

1.7.1. Descubrimiento

Como se mencionó previamente, los BoAstV fueron descritos por primera vez en el año 1978 junto con los BoNoV en muestras de heces de terneros diarreicos (Woode y Bridger, 1978).

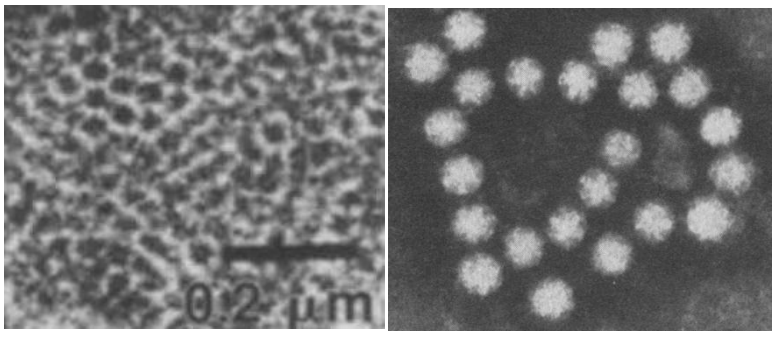


Figura 15. Micrografías electrónicas de partículas de astrovirus. Se pueden observar algunas partículas con forma de estrella de 5 o 6 puntas. Tomadas de Aroonprasert *et al*., 1989 y Bridger *et al*., 1984.

1.7.2. Enfermedad

Si bien el rol de los BoAstV en los casos de diarrea aún permanece controversial, hay evidencia de la asociación de los mismos en algunos brotes de DNT (Oem y An, 2014; Alfred *et al*., 2015; Nagai *et al*., 2015). BoAstV infecta células epiteliales de la cúpula del íleon sobre los parches de Peyer, mientras que no se ha observado infección en el intestino delgado o el grueso; por otro lado, se ha descrito que la infección por BoAstV es más severa en aquellos casos de coinfección con otros virus como torovirus y rotavirus (Woode *et al*., 1984). En los últimos años, los BoAstV han tomado mayor importancia debido al descubrimiento de BoAstV neurotrópicos, los cuales provocan encefalitis en aquellos animales infectados (Li *et al*., 2013; Bouzalas *et al*., 2014; Bouzalas *et al*., 2016; Schlottau *et al*., 2016; Selimovic-Hamza *et al*., 2017).

1.7.3. Características generales

En cuanto a la organización del genoma, la longuitud del mismo varía entre 6.253 y 6.317 nucleótidos, similar a otros astrovirus con tres ORFs solapados que codifican para las proteínas no estructurales y de la cápside (Tse *et al*., 2011). El ORF1a codifica para una proteasa, nsp1a, y tiene cerca del extremo 3' una secuencia "heptamérica resbaladiza" conservada (5'-AAAAAAC-3'), la cual es responsable de inducir un desplazamiento de los marcos ribosómicos durante la traducción para generar la poliproteína nsp1ab (Tse *et al*., 2011). El ORF1a y el ORF1b se solapan en una región de entre 102 y 147 nucleótidos de longitud. El ORF1b tiene un marco de lectura -1 en relación al ORF1a y codifica para una ARN polimerasa ARN dependiente. Cuando se consideran conjuntamente, ORF1a y ORF1b codifican una poliproteína nsp1ab clivada luego por la proteasa viral para convertirse en proteínas no estructurales activas. Por último, el ORF2 tiene entre 2.280 y 2.319 nucleótidos de longitud, y codifica las proteínas estructurales en el mismo marco de lectura que ORF1a (Tse *et al*., 2011). Hasta el momento se conocen dos serotipos distintos (Woode *et al*., 1985).

1.7.4. Clasificación

La clasificación actual según el ICTV para los astrovirus bovinos es: *Riboviria › Orthornavirae › Pisuviricota › Stelpaviricetes › Stellavirales › Astroviridae › Mamastrovirus.* Dentro del género *Mamastrovirus*, hay 19 especies confirmadas por el ICTV, siendo que los BoAstV no han sido oficialmente asignados a ninguna de ellas, aunque se ha propuesto que los BoAstV asociados a encefalitits pertenecen a MAstV-13 (Donato y Vijaykrishna, 2017). Además, se ha propuesto que pertencen a otras especies aún no reconocidas por el ICTV:

MAstV-24, MAstV-28, MAstV-29, MAstV-30 y MAstV-33 (Donato y Vijaykrishna, 2017).

1.7.5. Transmisión

Desafortunadamente, la vía de transmisión de BoAstV no está esclarecida, aunque posiblemente una vía sea la fecal-oral. Además, no está claro como logra infectar a nivel de sistema nervioso central.

1.7.6. Prevención

No hay vacuna comercial disponible para BoAstV, por lo que no hay, actualmente, una estrategia de prevención dirigida especialmente a este virus.

1.7.7. BoAstV en la región

Los datos publicados sobre BoAstV en la región son también muy escasos, contando únicamente con un artículo sobre la detección de este virus en Brasil, donde se observó una prevalencia de 14,3%. Además, se observó una asociación del virus con la presencia de diarrea, ya que la mayoría de las muestras positivas se obtuvieron de animales diarreicos (Candido *et al*., 2015). En Uruguay, no hay registros publicados sobre la presencia de este virus, por lo que no se toman medidas preventivas contra el mismo.

1.8. Virus en el ambiente

Si bien la virología ambiental como tal comenzó en 1940, fue luego de la mejora de los métodos de concentración, y principalmente con el desarrollo de la reacción en cadena de la polimerasa (PCR), que logró expandirse, tomando mucha relevancia en los últimos años (Miagostovich y Vieira, 2017).

Las enfermedades transmitidas por el agua se encuentran entre las causas más comunes de muerte en el mundo, y afectan especialmente a las sociedades más pobres y a los niños menores de 5 años en los países en desarrollo (Miagostovich y Vieira, 2017). Sin embargo, aún no ha sido extensamente aplicada más allá de virus que afectan a la salud humana, representando una carencia muy importante.

Los virus entéricos son a menudo objeto de investigación en el medio ambiente, incluidos todos los grupos virales que están presentes en el tracto gastrointestinal humano y que, después de la transmisión fecal-oral, pueden causar infecciones en individuos susceptibles. Son altamente resistentes a condiciones ambientales desfavorables que comparten propiedades, particularmente en lo que respecta al riesgo de enfermedades, como excreción en altas concentraciones incluso en casos asintomáticos, menor dosis infecciosa, estabilidad de partículas virales y resistencia a procedimientos de desinfección (Miagostovich y Vieira, 2017).

Los métodos de concentración más exitosos son aquellos basados en las propiedades del virus como adsorción/elución (carga iónica de la partícula viral), ultrafiltración (tamaño de partícula) y ultracentrifugación (densidad y coeficiente de sedimentación) asociados con el cultivo celular y ensayos moleculares para la recuperación de virus en diferentes matrices del medio ambiente. Si bien la detección del genoma viral no aporta información sobre la infectividad, la PCR, por su alta sensibilidad, especificidad y rapidez, se ha considerado una buena técnica para el monitoreo ambiental, especialmente por permitir la detección de virus que no se propagan en cultivo celular, y por lo tanto no pueden aislarse para su estudio exhaustivo. Además, la baja estabilidad del ARN libre en el medio ambiente sugiere que los métodos moleculares detectan partículas virales intactas, no genoma viral libre. Sin embargo, debido a que la infectividad está directamente relacionada con el riesgo para la salud, se han seleccionado metodologías capaces de distinguir partículas infecciosas y no infecciosas, como el ensayo de cultivo celular integrado a la PCR (ICC-PCR), la detección de ARNm viral y protocolos con tratamiento enzimático para evaluar muestras ambientales (Miagostovich y Vieira, 2017).

La temperatura, la luz ultravioleta (UV), las sales, la materia orgánica, las interfaces aire-agua y los factores biológicos como la microflora acuática son factores importantes para controlar la supervivencia de los virus entéricos en el agua que generalmente permanecen estables durante meses o más cuando se asocian con partículas en agua y sedimentos (Miagostovich y Vieira, 2017).

Generalmente se utilizan indicadores bacterianos para el control microbiológico convencional de la calidad del agua. Sin embargo, esos indicadores presentan algunas limitaciones, como la sensibilidad a los métodos de inactivación en comparación con otros patógenos, así como la capacidad de multiplicarse en algunos entornos. También se han reportado enfermedades virales transmitidas por el agua o los alimentos cuando esas matrices cumplen con los estándares bacterianos vigentes, generando un consenso global sobre la falta de correlación entre la contaminación bacteriana y la presencia viral (Miagostovich y Vieira, 2017).

En los tambos, los terneros suelen ser criados en guacheras, donde se los alimenta con agua, así como también se utiliza agua para preparar los sustitutos lácteos en los casos que los utilizan (Hötzel *et al*., 2014). El agua que se utiliza para el consumo de los terneros suele obtenerse de cursos de agua subterráneos, a través de pozos. Además, muchas veces para el tratamiento de la DNT suelen utilizarse soluciones de electrolitos preparadas con agua, así como también se recomienda la disponibilidad de agua para los terneros afectados para evitar la deshidratación (McGuirk, 2008). Por otra parte, la calidad del agua, así como de los contenedores en los cuales se deposita la misma para el consumo de los terneros, suele estar dirigido a la búsqueda de bacterias, sin tener en cuenta la posible contaminación viral (McGuirk, 2008).

Hasta el momento, no se cuenta en la literatura, con trabajos que evalúen la contaminación viral de las aguas utilizadas para el consumo de los terneros.

1.9. Relevancia

De lo expuesto previamente se desprende la importancia y necesidad de conocer los virus que afectan la producción ganadera tanto de carne como de leche en nuestro país, y por tal motivo el presente trabajo de tesis tuvo como objetivo determinar la frecuencia de detección y caracterizar molecularmente cepas de RVA, BCoV, BoNoV, BToV y BoAstV presentes en muestras clínicas y ambientales de rodeos de carne y leche del Uruguay.

2. Hipótesis

Esta tesis tuvo como hipótesis que los virus entéricos bovinos están presentes y circulando en el ganado vacuno en nuestro país.

3. Objetivos

3.1. Objetivo general: Caracterizar a nivel molecular y estimar la prevalencia de virus entéricos bovinos en muestras fecales de terneros para producción de carne y leche, y en aguas de consumo en tambos de Uruguay.

3.2. Objetivos específicos:

- 3.2.1. Desarrollar metodologías moleculares para la detección y caracterización molecular de RVA, BCoV, BoNoV, BToV y BoAstV.

- 3.2.2. Determinar el grado de contaminación viral de las aguas utilizadas para el consumo de los terneros en las principales zonas tamberas del país.

- 3.2.3. Estimar la prevalencia de los virus mencionados en casos de diarrea neonatal de terneros.

- 3.2.4. Establecer cuáles son los principales genotipos, subtipos y linajes, de cada uno de estos patógenos virales circulantes en rodeos de carne y leche del Uruguay.

- 3.2.5. Estimar la distribución temporal, espacial y geográfica de las infecciones virales que causan diarrea neonatal en terneros en las principales zonas tamberas del Uruguay.

- 3.2.6. Estudiar a estos virus como posibles causas de muerte de terneros.

- 3.2.7. Determinar el grado de variabilidad genética para los virus mencionados, a partir de las estirpes detectadas en Uruguay y su comparación con aquellas de la región y del resto del mundo.

- 3.2.8. Realizar estudios comparativos entre las estirpes detectadas en campo y las utilizadas en las vacunas existentes en el país para RVA y BCoV.

4. Materiales, métodos, resultados y discusión

Los materiales y las metodologías utilizadas, así como también los resultados y la discusión de los mismos, están descritas en cada artículo publicado, o bajo revisión.

Los artículos se muestran ordenados de la siguiente manera: en primer lugar la sección ambiental de la tesis, y luego la sección clínica con los artículos ordenados por virus y no cronológicamente para una mejor comprensión. Entonces, la sección clínica está ordenada de la siguiente manera: en primer lugar BCoV y RVA, que son los virus más estudiados y asociados a DNT, y luego BoAstV, BoNoV y BToV, cuyo papel en la DNT ha sido secundario, debido principalmente a que se han estudiado en menor medida.

A. Sección de estudios de virus a partir de muestras ambientales

A.1. Virus en el ambiente

Esta tesis pretendió abordar el estudio de los virus entéricos bovinos globalmente, tanto en muestras clínicas como ambientales. El estudio de los virus en el ambiente es importante porque no solamente nos permite conocer el grado de contaminación viral que hay en los establecimientos ganaderos de nuestro país, sino que también muchas veces puede ser el origen de brotes de DNT, incluso cuando se han tenido precauciones en el manejo, no ha habido introducción de animales al establecimiento, ni hay animales con diarrea, por lo que el inicio del brote es inesperado. El estudio de los virus en el ambiente ha tomado relevancia en los últimos años, focalizándose principalmente en diferentes tipos de agua tanto para consumo humano, recreación, entre otros usos, pero no se contaba (al menos hasta donde llegaba nuestro conocimiento) con estudios que analizaran el grado de contaminación de las aguas utilizadas para el consumo de los terneros.

La sección ambiental trató de cumplir con dos de los objetivos de esta tesis: el 3.2.1., el cual pretendía desarrollar metodologías moleculares para la detección de los virus en diferentes tipos de muestra, en este caso muestras ambientales, y el 3.2.2., específico de esta sección ambiental, el cual pretendía determinar el grado de contaminación viral de las aguas utilizadas para el consumo de los terneros en las principales zonas tamberas del país.

En este sentido, un artículo principal fue publicado, el cual estuvo enfocado en determinar la prevalencia y la viabilidad de RVA (utilizado como indicador de contaminación viral) en aguas utilizadas para el consumo de los terneros en los tambos.

A.2. Estudio de la contaminación viral en aguas utilizadas para el consumo de terneros en tambos de Uruguay

Artículo 1. Prevalencia y viabilidad de rotavirus del grupo A en fuentes de agua de tambos

Este artículo permitió demostrar el grado de contaminación de RVA de las aguas utilizadas para el consumo de los terneros en los tambos de nuestro país. Se analizaron tanto muestras de agua tomadas directamente de los pozos, así como también muestras de agua tomadas de los bebederos, siendo que se detectaron muestras positivas en ambos tipos de muestra. El 35% (71/202) de las muestras analizadas fue positiva a RVA; 44% (56/127) en aguas de bebederos y 20% (15/75) en aguas de pozo. Se observó un marcado patrón de estacionalidad, con mayor frecuencia de detección en los meses más fríos. Además, se pudo comprobar que las partículas virales detectadas en estas muestras se mantenían viables, por lo tanto, con su capacidad infectiva intacta, pudiendo ser la transmisión hídrica una vía de transmisión del virus. Por último, se determinó que la causa más probable de inicio de un brote de diarrea asociada a RVA fue el agua, ya que se detectó el virus previo al inicio del mismo, en el agua utilizada para el consumo de los terneros en el establecimiento afectado.

Estos resultados fueron publicados en la revista Journal of Applied Microbiology: **Castells M**, Schild C, Caffarena D, Bok M, Giannitti F, Armendano J, Riet- Correa F, Victoria M, Parreño V, Colina R. Prevalence and viability of group A rotavirus in dairy farm water sources. J Appl Microbiol. 2018 Mar;124(3):922-929. doi: 10.1111/jam.13691.

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ORIGINAL ARTICLE

Prevalence and viability of group A rotavirus in dairy farm water sources

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Abstract

Aim: To analyse group A rotavirus (RVA) environmental contamination in waters used for calves' consumption and to assess viral viability in dairy farm water sources.

Methods and Results: We analysed 202 samples of water used for calves' consumption and RVA was detected by RT-qPCR in 35·1% (95% CI: 28·9–42·0%). A marked pattern of seasonality was observed with higher frequency of detection in colder than warmer months ($P = 0·002$). There was no association between viral load and season or between the number of milking cows in the herd and the detection of RVA in the farm. The viability of the RVA particles detected was confirmed by isolation of RVA in cell culture from 5 of 10 water samples. Furthermore, an RVA waterborne outbreak of neonatal calf diarrhoea was described.

Conclusions: We demonstrate that RVA is frequent in dairy farm waters, and that the virus is infectious and capable of generating a diarrhoea outbreak.

Significance and Impact of the Study: Neonatal diarrhoea syndrome leads to economic losses to the livestock industry worldwide. To determine transmission routes is essential to take action in this regard and reduce the impact that this syndrome has for the livestock production. The results obtained in this work alert the dairy industry and highlight that mitigation strategies are crucial to improve the microbiological quality of this water.

Introduction

Neonatal diarrhoea is a main syndrome of worldwide distribution affecting humans and animals, and can result in high morbidity and mortality (Estes and Greenberg 2013). Neonatal calf diarrhoea has a negative impact on animal welfare and leads to economic losses to the livestock industry due to cost of treatment, prophylaxis, increased susceptibility to other diseases, mortality and long-term residual effects, such as reduced growth rates and milk production (Waltner-Toews *et al.* 1986; Donovan *et al.* 1998; Østerås *et al.* 2007; Windeyer *et al.* 2014). In some areas, neonatal calf diarrhoea can be associated with >50% of calves' deaths (USDA, 2008; Hur *et al.* 2013). Group A rotavirus (RVA) is a main pathogen associated with the neonatal diarrhoea syndrome (Izzo *et al.* 2011; Al Mawly *et al.* 2015). To decrease both the presence of RVA and the severity of the disease, recommendations are mainly aimed at improving cattle management, hygiene and sanitation, decreasing pathogen exposure, and reducing pathogen load in the environment (Cho and Yoon 2014). Vaccination of pregnant cows coupled with adequate colostrum management practices to infer passive transfer of immunity, is the main strategy for prevention of RVA diarrhoea in newborn calves, generally reducing viral shedding or diminishing the disease symptoms and deaths (Saif *et al.* 1983; Fernandez *et al.* 1998; Parreño *et al.* 2004, 2010).

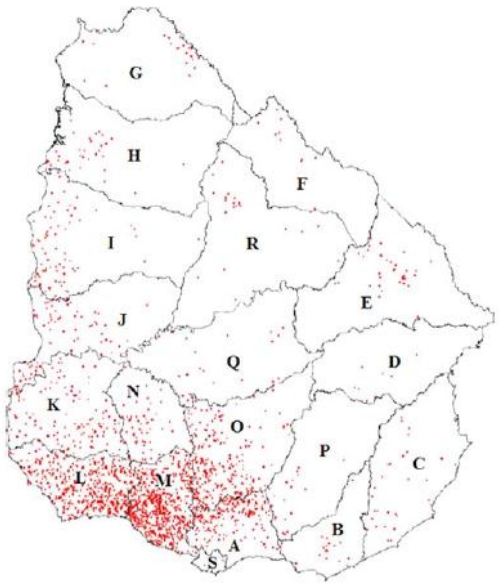

Figure 1 Map of dairy farms in Uruguay. Each red dot represents two dairy farms. Letters indicate each of the 19 departments in which Uruguay is divided. (A) Canelones, (B) Maldonado, (C) Rocha, (D) Treinta y Tres, (E) Cerro Largo, (F) Rivera, (G) Artigas, (H) Salto, (I) Paysandú, (J) Río Negro, (K) Soriano, (L) Colonia, (M) San José, (N) Flores, (O) Florida, (P) Lavalleja, (Q) Durazno, (R) Tacuarembó and (S) Montevideo. Most dairy farms are concentrated in the southwestern region of the country (modified from DIEA, 2016). [Colour figure can be viewed at wileyonlinelibrary.com]

Group A rotavirus is widespread worldwide and its presence in different aquatic matrices is well documented (Odagiri *et al.* 2016; De Giglio *et al.* 2017; Delgado-Gardea *et al.* 2017; Tort *et al.* 2017). High excretion levels and low minimal infecting dose make enteric viruses easily transmissible (Estes and Greenberg 2013).

In Uruguay, the dairy industry is one of the main economic sectors with almost 4000 dairy farms and 783 000 cattle heads, located principally in the southwest region of the country (Fig. 1) (DIEA, 2016). Calves in Uruguayan dairy farms are raised either individually or, quite frequently, in collective pens where many calves share the same space, feeders and water containers (water troughs). Furthermore, water used for dissolving powdered milk replacers fed to calves during the first weeks of life, comes from underground water wells and is generally not subjected to any type of water purification or decontamination treatment. In addition, water quality used for cattle consumption is very poorly studied. The aim of this study was to determine the prevalence of RVA in waters used for calves' consumption from the main dairy basin in Uruguay, and to assess viral viability in a subset of RVA-positive samples. To our knowledge, this is the first comprehensive study about RVA prevalence and viability in water wells and troughs, used for calves' consumption.

Materials and methods

Study area and sample collection

The sampling strategy was designed to collect water from the farms randomly, considering the number of dairy farms in each department (Canelones, Colonia, Florida, Maldonado, Paysandú, Río Negro and San José) (Fig. 1). Samples were collected in the four defined climate seasons in Uruguay: winter (21st June–20th September), spring (21st September–20th December), summer (21st December–20th March) and autumn (21st March–20th June).

A total of 202 water samples used for calves' consumption were collected from July 2015 to June 2016 in 192 dairy farms; 127 of the samples were obtained directly from water containers (water troughs) of collective rearing calf pens, and the remaining 75 from underground water wells. Volume collected for each sample was 500 ml, samples were maintained at 4°C, which were immediately transported to the laboratory and processed.

Water sample concentration, recovery, nucleic acid extraction, reverse transcription and quantitative polymerase chain reaction

The viral concentration method used was adsorption–elution with a negative charged membrane (Haramoto *et al.* 2009). Briefly, 5 ml of 2·5 mol l^{-1} magnesium chloride (final concentration of 25 mmol l^{-1}) was added to 500 ml of each sample, and filtered in HA electronegative membranes (Millipore®, Burlington, MA, USA) with pore size of 0·45 μm. Then, membranes were rinsed with 50 ml of 0·5 mmol l^{-1} sulphuric acid (pH 3·0). To elute the viral particles, 4 ml of sodium hydroxide 1 mmol l^{-1} (pH 10·5) was added to the membranes and mixed/shaken for 5 min. Finally, the solution was neutralized with 40 μl of 50 mmol l^{-1} sulphuric acid and 40 μl of 100X Tris-EDTA buffer (pH 8·0). To determine the recovery rate of the process, two water samples from water containers (previously determined as negative samples for RVA) were inoculated with 500 μl of 10% faecal suspension of two neonate calves with known concentration of RVA, previously determined by quantitative polymerase chain reaction (qPCR).

Viral RNA was extracted using QIAamp® cador® Pathogen Mini Kit (Qiagen®, Hilden, Germany), following the manufacturer's instructions with an elution volume of 50 μl. Reverse transcription (RT) was carried out with RevertAid Reverse Transcriptase (Thermo Fischer Scientific®, Waltham, MA, USA) and random hexamer primers (Qiagen). Ten microlitres of RNA and 10-fold dilution of RNA (per RNA) were used as template to reduce inhibitors concentration, and RNA and cDNA were stored at −20°C. qPCR was performed with TaqMan® technology and

Rotor-Gene Q instrument (Qiagen); undiluted and 10-fold diluted samples were analysed in duplicate. In order to quantify RVA, a standard curve was generated by using 10-fold dilutions (10^7–10^0 genome copies per μl) of a plasmid containing an amplicon of 86 bp described by Zeng *et al.* (2008). Five microlitres of cDNA as template, SensiFAST™ Probe No-ROX Kit (Bioline®, London, UK), primers and probes described in Zeng *et al.* (2008) with a final concentration of 0·4 and 0·2 μmol l^{-1}, respectively, and DNase/RNase-free water to a final volume of 25 μl, were used.

Statistical analyses

Data were analysed by using generalized linear models (GENMOD procedure, SAS Studio ver. 3.6; SAS Institute Inc., Cary, NC). For the analysis of the percentage of positive samples, a binomial distribution of the variable and a logit link function were assumed. Season and number of milking cows were evaluated as fixed effects in different univariable models. The analysis was performed separately for water container samples, water well samples, and for the total number of samples. For the analysis of the viral load in positive samples, the variable was $\log_{10}$ transformed and analysed in the same way as for the percentage of positive samples; a gamma distribution of the variable and a logarithmic link function were assumed. Results are expressed as least square means ± standard error (SE) with its respective 95% confidence intervals ($CI_{95\%}$).

Viral viability in water samples

To assess the infectivity of RVA detected by qPCR, the 10 samples with the highest viral load were tested in cell culture (Rutjes *et al.* 2009). Briefly, MA-104 cells were grown in 24-well tissue culture plates (CellStar®; Greiner Bio One, Kremsmünster, Austria) at 37°C in 5% CO_2 atmosphere until monolayers reached 80–100% of confluence. Before inoculation, 200 μl of water samples were clarified with 200 μl of chloroform (1 : 1 ratio). Each well was inoculated with 50 μl of water sample and 50 μl of Dulbecco's modified eagle's medium (DMEM) supplemented with 1% of antibiotic–antimycotic solution (Anti-anti; Gibco®, Thermo Fischer Scientific, Waltham, MA, USA) and trypsin at a final concentration of 2 μg ml^{-1}. The 24-well tissue culture plate containing cells and inoculums were incubated for 1 h at 37°C. Afterwards, the inoculums were removed and each well overlaid with 500 μl of DMEM supplemented as mentioned above and incubated for 4 days at 37°C and 5% CO_2 atmosphere, on four consecutive passages of 4 days each. During each passage, cell monolayers were observed under optical microscope for cytopathic effect (CPE), and prefixed monolayers were revealed by direct immunofluorescence using a 3B2 monoclonal VHH nanoantibody directed to VP6 of RVA (Garaicoechea *et al.* 2008) conjugated with Alexa Fluor (Alexa 488; Invitrogen®, Thermo Fischer Scientific, Waltham, MA, USA). qPCR was performed for all passages from the 10 samples (n = 40) as mentioned above.

Waterborne outbreak study

In September 2015, a spontaneous neonatal diarrhoea outbreak affected a collective calf pen in a dairy farm in Colonia, Uruguay. A water sample was collected a month before the outbreak, and 12 faecal and 1 water samples were collected during the outbreak. Faecal suspensions were prepared at a dilution of 10% in PBS and processed as described above for viral RNA extraction, RT and RVA qPCR.

Results

Recovery rate of the process

The mean recovery rate of the complete process (from viral concentration to qPCR result) was 13·17 ± 10·31% (mean ± standard deviation); the two observed recovery rates were 5·88 and 20·46%, and no significant differences were obtained between pure and 10-fold dilutions (data not shown).

Frequency of RVA detection and viral concentration in water

Overall, RVA genomes were detected in 71 of the 202 samples (35·1%). In water containers, RVA was detected

Table 1 Frequency of detection of RVA and viral load in water samples

		RVA-positive samples				Viral load in positive samples ($\log_{10}$ gc per l)			
Source	*n*	*n*	Percentage	SE	$CI_{95\%}$	Mean	SE	$CI_{95\%}$	Observed range
Water containers	127	56	44·1	4·4	35·8–52·8	5·8	0·2	5·4–6·1	4·5–9·6
Water wells	75	15	20·0	4·6	12·5–30·4	5·3	0·2	4·8–5·8	3·8–7·6
Total	202	71	35·1	3·4	28·9–42·0	5·7	0·1	5·4–5·9	3·8–9·6

n, number of samples; SE, standard error; $CI_{95\%}$, 95% confidence interval.

in 56 of 127 samples (44·1%), while 15 of 75 (20·0%) underground water wells were positive (Table 1). The overall mean viral concentration was 5·7 $\log_{10}$ genome copies per litre ($\log_{10}$ gc per litre); the mean concentration in the water containers was 5·8 $\log_{10}$ gc per litre, whereas in the water wells it was 5·3 $\log_{10}$ gc per litre. The observed range, SE and $CI_{95\%}$ are shown in Table 1.

The percentage and absolute number of positive water samples for each department sampled was: 20% in Canelones (3 of 15), 33% in Colonia (7 of 21), 28% in Florida (17 of 61), 50% in Maldonado (1 of 2), 11% in Paysandú (1 of 9), 19% in Río Negro (3 of 16) and 40% in San José (20 of 50). Inhibition was not observed when pure and 10-fold dilutions were compared (data not shown).

Seasonality of RVA in water samples

Seasonality of RVA detection was assessed in this 1-year study, determining the frequency of RVA detection in the four seasons, and comparing between colder (autumn and winter) and warmer (spring and summer) seasons (Fig. 2). Of the total number of samples analysed, the percentage of detection was 50% (8 of 16) during autumn, 47% (34 of 73) during winter, 26% (6 of 23) during summer and 26% (23 of 90) during spring. In the warmer seasons, the overall percentage of detection was 26% (29 of 113), whereas in the colder seasons the percentage was 47% (42 of 89); a marked pattern of seasonality was observed with higher frequency of detection in colder than warmer months ($P = 0{\cdot}002$). This seasonality was also observed when the two sample types (water containers and underground water wells) were analysed separately. Water wells showed a percentage of detection of 40% (10 of 25) in winter, 14% (1 of 7) in summer, 10% (4 of 39) in spring and 0% (0 of 4) in autumn. Samples collected in colder months showed a higher frequency of RVA detection (34%, 10 of 29) than those collected in warmer months (11%, 5 of 46, $P = 0{\cdot}014$). When water containers were analysed, autumn was the season with the highest frequency of detection (67%, 8 of 12), followed by winter with (50%, 24 of 48), spring (37%, 19 of 51) and summer (31%, 5 of 16). Again, a higher frequency of detection was observed in the colder months (53%, 32 of 60) when compared with the warmer months (36%, 24 of 67, $P = 0{\cdot}047$). No statistically significant differences were found between the viral load and the different seasons (Fig. 3).

Herd size and detection of RVA

Dairy farms were evaluated according to the number of milking cows in small (<100), medium (100–299) and

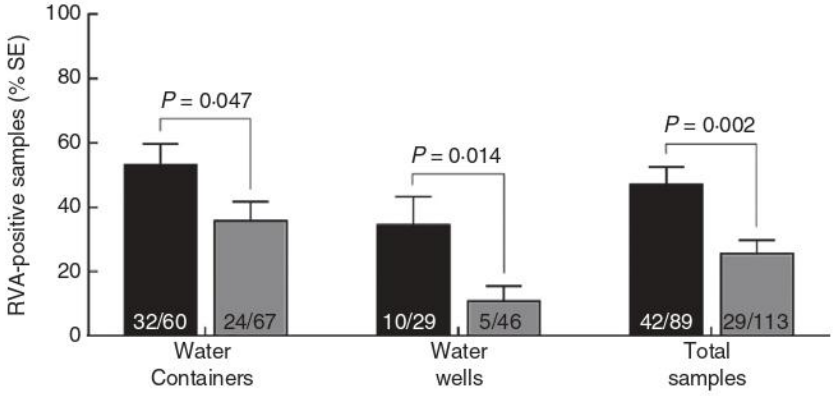

Figure 2 Seasonal distribution of group A rotavirus (RVA). Percentage of RVA positivity samples according with the temperature of the season and water source (autumn–winter—colder seasons; and spring–summer—warmer seasons). Dark columns: autumn–winter, light columns: spring–summer.

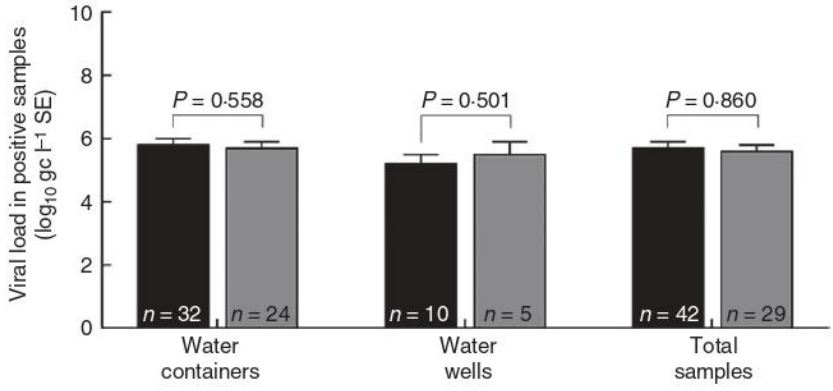

Figure 3 Viral load in water samples and seasonal temperature. The viral load according to the temperature of the season was compared for the water containers, the water wells and the total samples (autumn–winter—colder seasons; spring–summer—warmer seasons). Dark columns: autumn–winter, light columns: spring–summer.

large (>299) herds. No statistical association was found between the herd size in each farm and the frequency of RVA detection or the viral load in the water samples (Tables S1 and S2).

Viability of RVA detected in water containers

We confirmed the cell culture infectivity of viral particles in 5 of 10 water samples studied; two of them were positive by direct immunofluorescence and ICC-RT-qPCR, and showed CPE after second passage (Fig. S1), whereas three of them were only detected by ICC-RT-qPCR after fourth passage.

Waterborne outbreak of calf diarrhoea associated with RVA infection

Eleven of the 12 faecal samples and both water samples of water containers (pre- and intra-outbreak) were positive for RVA. The mean viral load in the faecal samples was 8·2 $\log_{10}$ gc per ml, ranging between 5·0 and 12·2 $\log_{10}$ gc per ml. The viral loads in the water samples were 6·9 and 7·2 $\log_{10}$ gc per litre, for pre- and intra-outbreak respectively.

Discussion

Neonatal calf diarrhoea is a major problem for the livestock industry, leading to economic losses due to cost of treatment, long-term difficulties such as reduced weight and milk production, and calf mortality (Waltner-Toews *et al.* 1986; Donovan *et al.* 1998). As RVA is one of the major pathogens involved in neonatal calf diarrhoea (Izzo *et al.* 2011; Al Mawly *et al.* 2015), we studied the presence and infectivity of RVA in waters used for calves' consumption in dairy farms. We first determined the recovery efficiency of the concentration method in our aquatic matrix; the results (5·88–20·46%) were similar to those obtained with river and pond water described in the reference method study (Haramoto *et al.* 2009) as well as in other studies (Fumian *et al.* 2010; De Keuckelaere *et al.* 2013). The adsorption–elution method using a negative charged membrane is useful for determining virus concentration in water samples from water containers, but it is worth mentioning that some samples present difficulties to pass through the membrane mainly due to contamination with grass, dirt and saliva.

We detected a high frequency of RVA in the analysed samples (35%), which indicates a widespread distribution in the country and suggests a risk of RVA transmission to calves that drink these waters. When the source of water was analysed separately, we observed the highest frequency of detection (44%) in the water containers. Although this is the first study in this type of matrix, the results were expected because this water originally came from water wells but, while in the containers, it was exposed to potential sources of direct contamination, such as cattle, wildlife and calves shedding the virus. When water well samples (groundwater) were analysed, RVA was detected in 20% of the samples, a frequency comparable to that reported by other authors with RVA detection up to 40% (Jung *et al.* 2011; Ferguson *et al.* 2012; Spilki *et al.* 2014; De Giglio *et al.* 2017). Particularly, Spilki *et al.* (2014) showed a frequency of RVA detection of 6·7% from a creek, but there was no detection of RVA in the groundwater samples of dairy farms. The results obtained herein demonstrate a high frequency of RVA detection in the groundwater of dairy farms which represents a potential risk not only for animals but also for humans living in these farms, given that it is also common to use water from these wells for human consumption.

There was a variation in the frequency of RVA detection among departments. Maldonado showed the highest proportion of positive samples, but due to the very few samples available from this department (two samples) this result is most likely overestimated. On the other hand, San José showed the highest number of RVA-positive water samples (20) and a high percentage (40%). Of the three major departments with larger numbers of dairy farms (Colonia, Florida and San José), Florida showed the lowest frequency of detection (28%).

The mean viral load was similar for the two sources of water analysed; 5·8 and 5·3 $\log_{10}$ gc per litre for water containers and water wells respectively. We found high viral concentration (>6·0 $\log_{10}$ gc per litre) in 17 water containers, generally from farms suffering diarrhoea outbreaks (data not shown) at the time of sample collection, and in two water wells, one of which corresponded to a sample collected in Paysandú on December 2015. This area had heavy rainfalls, with one of the largest floods of the Uruguay river ever recorded, around the date of sample collection. It is important to stress that the main sources of contamination for the groundwater are animal faeces or wastewater due to the rainfall (Abbaszadegan *et al.* 2003; Masciopinto and Visino 2017; Moreira and Bondelind 2017). Regarding water container contamination, we observed a wide range of viral load (4·5–9·6 $\log_{10}$ gc per litre). Probably, the lowest concentrations are a result of the contamination from the groundwater used to fill them up, and the higher concentrations are from direct contamination with faeces of infected cattle, which is in concordance with the results obtained for the percentage of detection previously mentioned.

It is interesting to note that the seasonality pattern with peaks of RVA detection in colder months was observed

both in water wells and water containers. The seasonality in groundwater likely reflects the environmental contamination, and the seasonality in water containers is probably secondary to both environmental and faecal contamination from cattle. It is documented that RVA infections and the presence of the virus in the environment are more common in colder months (Ho *et al.* 1988; Matson *et al.* 1990; Chan *et al.* 2013; Corsi *et al.* 2014; Victoria *et al.* 2014), therefore, our results are in agreement with these works.

In this work, the viability of the viral particles was assessed only in the water container samples because the viability of RVA in groundwater is well documented; however, to our knowledge, viral viability in the water from water containers has not been explored. Here, we demonstrate the infectivity of RVA particles in 5 of 10 water samples. We could not determine whether the five negative samples were due to the lack of infective particles in the sample or if the viral strains present did not adapt to the cell culture, given that RVA generally present difficulties (are 'fastidious') to adapt to cell culture (Hasegawa *et al.* 1982; Wyatt *et al.* 1983), particularly from environmental samples (Rutjes *et al.* 2009). Nevertheless, the detection of infective viral particles in five samples, confirms the risk of infection in calves consuming these waters. This is crucial information that farmers should consider in the decision making when managing the cattle. The implementation of a water treatment to inactivate this and other pathogens before administering it to calves is advised; the treatment of water in rural areas is strongly recommended (Odagiri *et al.* 2016).

Lastly, we suspect that an outbreak of diarrhoea in a dairy farm with detection of RVA in 11 of 12 calves, was a waterborne outbreak. The detection of RVA in pre- and intra-outbreak water samples obtained from the water container from which the calves consumed the water, but particularly the detection of RVA in the pre-outbreak water sample, indicates that this could have been the initial source of infection. Unfortunately, we could not obtain the sequences of RVA in the water samples, in order to confirm that RVA circulating in the outbreak was the same that was present in the water.

To the best of our knowledge, this is the first study evidencing the presence of one of the most important enteric viruses causing neonatal diarrhoea such as RVA in waters used directly for calves' consumption. We demonstrate that RVA is highly prevalent in the water used for calves' consumption in dairy farms, and that RVA in these samples is infective and potentially capable of generating a neonatal diarrhoea outbreak under field conditions. The detection of RVA was associated with lower temperatures, whereas the herd size did not affect the frequency of detection. Our results highlight that mitigation strategies are needed to improve the microbiological quality of the water used for dairy cattle farming.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Viability of RVA particles in waters from containers.

Table S1. Herd size and frequency of RVA detection in water samples

Table S2. Herd size and viral load in water samples

Table S1. Herd size and frequency of RVA detection in water samples.

Source	MC 33 – 99				MC 100 – 299				MC > 300				P-Value
	n	RVA-Positive samples			n	RVA-Positive samples			n	RVA-Positive samples			
		n	% ± SE	$CI_{95\%}$		n	% ± SE	$CI_{95\%}$		n	% ± SE	$CI_{95\%}$	
Water containers *	24	6	25.0 ± 8.8	11.7 – 45.6	24	10	41.7 ± 10.1	24.1 – 61.7	36	17	47.2 ± 8.3	31.7 – 63.3	0.205
Water wells **	6	1	16.7 ± 15.2	2.3 – 63.1	11	4	36.4 ± 14.5	14.3 – 66.1	34	4	11.8 ± 5.5	4.5 – 27.4	0.215
Total	30	7	23.3 ± 7.7	11.6 – 41.5	35	14	40.0 ± 8.3	25.3 – 56.7	70	21	30.0 ± 5.5	20.5 – 41.7	0.338

n: number of samples, SE: standard error, CI95%: 95% confidence interval, MC: milking cows

* MC missing for 43 samples (23 positive samples)

** MC missing for 24 samples (6 positive samples)

Table S2. Herd size and viral load in water samples.

Source	Viral load in positive samples (log_{10} gc l^{-1})									P-Value
	MC 33 – 99			MC 100 – 299			MC > 300			
	n	% ± SE	$CI_{95\%}$	n	% ± SE	$CI_{95\%}$	n	% ± SE	$CI_{95\%}$	
Water containers *	6	5.1 ± 0.3	4.5 – 5.8	10	5.5 ± 0.3	5.0 – 6.1	17	5.6 ± 0.2	5.2 – 6.0	0.462
Water wells **	1	4.8 ± 0.4	4.1 – 5.7	4	5.1 ± 0.2	4.7 – 5.5	4	5.7 ± 0.2	5.2 – 6.1	0.153
Total	7	5.0 ± 0.3	4.5 – 5.6	14	5.4 ± 0.2	5.0 – 5.8	21	5.6 ± 0.2	5.2 – 5.9	0.284

n: number of samples, SE: standard error, CI95%: 95% confidence interval, MC: milking cows

* MC missing for 23 positive samples

** MC missing for 6 positive samples

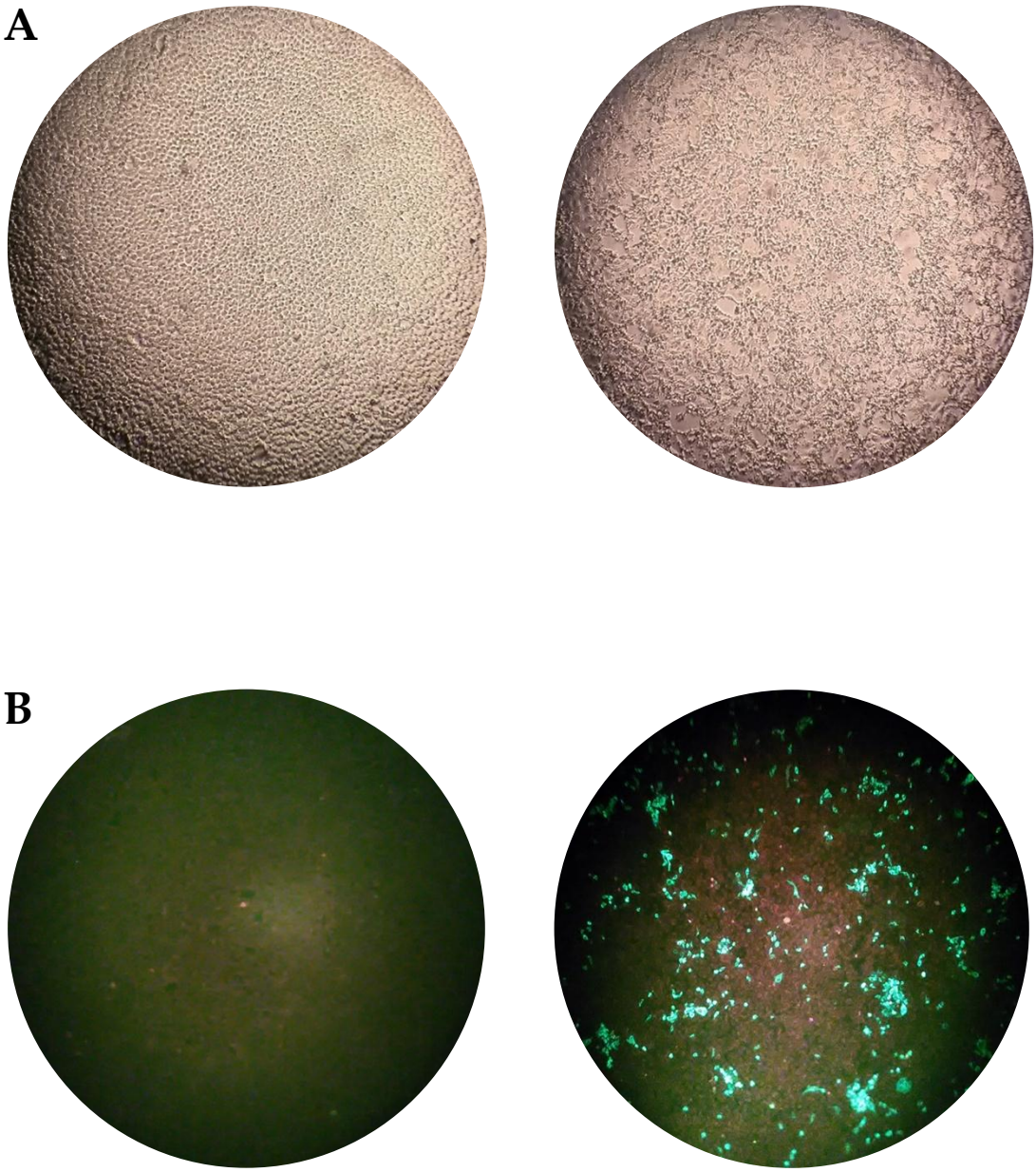

Fig. S1. Viability of RVA particles in waters from containers. A) Cytopathic effect of RVA observed in the 10X optical microscope. Left: negative control, right: positive sample. B) Direct immunofluorescence with 3B2 monoclonal VHH nanoantibody directed to VP6 of RVA labelled with 488 Alexa Fluor, observed in a 10X fluorescent microscope. Left: negative control, right: positive sample.

B. Sección de estudios de virus a partir de muestras clínicas

B.1. Virus en terneros

Esta sección se enfocó en el estudio de 5 virus asociados a DNT, o al menos con la capacidad de infectar a nivel intestinal. Dos de los virus estudiados, RVA y BCoV, están ampliamente estudiados, y asociados a DNT, siendo de los patógenos más prevalentes a nivel mundial, así como también a nivel regional. La inclusión de los otros 3 virus, se debió a que muchas veces han sido subestimados, y en el caso de BToV y BoNoV están asociados a DNT, siendo que BToV suele estar asociado a casos severos, mientras que BoAstV es un virus que está muy poco estudiado, pero ha tomado mucha relevancia ya que en los últimos años se lo ha asociado a casos de muerte por encefalitis.

Por otra parte, para la prevención de RVA y BCoV, para los cuales a pesar de no contar con trabajos publicados en revistas científicas internacionales, se conocía de su presencia en nuestro país, hay vacunas disponibles en el mercado. Esto algunas veces tiene un efecto secundario, que es que otros virus logran ocupar ese "nicho" que dejan estos virus, como se ha visto en los humanos, que luego de la vacunación contra RVA (obligatoria en muchos países), norovirus logra ocupar ese lugar, aumentando su circulación. En este sentido, es importante conocer cuáles son los virus que circulan en los bovinos de nuestro país, para poder comprenderlos y estar preparados para prevenirlos o combatirlos.

La sección clínica trató de cumplir con siete de los objetivos de esta tesis: el 3.2.1., el 3.2.3., el 3.2.4., 3.2.5., 3.2.6., 3.2.7., y 3.2.8.. Estos objetivos estaban dirigidos a tener optimizadas metodologías para detectar eficazmente los posibles virus asociados a DNT, así como también determinar la frecuencia de circulación de los mismos, factores de riesgo, las distintas variantes de cada virus, así como estudios evolutivos para poder comprender en mayor medida a estos virus que causan pérdidas económicas en nuestro país, con el objetivo de utilizar esta información para tomar medidas paliativas, e intentar colaborar con el sector productivo, lo cual redunda en un beneficio a nivel de país.

En este sentido, se publicaron 6 artículos principales, los cuales estuvieron dirigidos a comprender profundamente BCoV, RVA, BoAstV, y BoNoV. Además, otro artículo principal se encuentran en proceso de publicación en una revista arbitrada internacional, enfocado en el estudio de BToV. Por otra parte, otros 5 artículos vinculados a esta tesis, aunque de forma indirecta, fueron publicados o se encuentran en proceso de revisión. Estos trabajos cuentan con datos generados en el marco de esta tesis, principalmente vinculados a BCoV y RVA, en los cuales participé activamente.

B.2. Estudio de coronavirus bovino en muestras clínicas y análisis evolutivos

Artículo 2. Coronavirus bovino en Uruguay: diversidad genética, factores de riesgo e introducciones transfronterizas desde países vecinos

Este artículo estuvo enfocado principalmente en comprender la epidemiología de este virus en nuestro país, determinando la frecuencia de circulación, estudiando diferentes factores de riesgo, su diversidad genética, y análisis evolutivos que permitieron determinar cuándo y desde donde ingresó el virus a nuestro país. El virus fue detectado tanto en muestras de terneros para producción de carne como de leche, así como también en muestras de terneros vivos como de terneros muertos. Se observó que la vacunación es un método efectivo para controlar la circulación del virus, así como las bajas temperaturas son un factor de riesgo que aumenta la frecuencia de detección del virus en terneros. Por otra parte, se observó una divergencia importante a nivel filogenético entre las cepas circulantes con respecto a la cepa de referencia vacunal, CoV B Mebus, 1969, lo que alerta sobre el mantenimiento de la vigilancia para detectar posibles cepas divergentes que puedan escapar a la protección a través de la vacuna, y en ese caso incorporarlas a la formulación de la vacuna de forma temprana. Por último, se determinó que las dos variantes genéticas que circulan actualmente en nuestro país ingresaron en el año 2013, una desde Argentina y la otra desde Brasil.

Estos resultados fueron publicados en la revista Archives of Virology: **Castells M**, Giannitti F, Caffarena RD, Casaux ML, Schild C, Castells D, Riet-Correa F, Victoria M, Parreño V, Colina R. Bovine coronavirus in Uruguay: genetic diversity, risk factors and transboundary introductions from neighboring countries. Arch Virol. 2019b Nov;164(11):2715-2724. doi: 10.1007/s00705-019-04384-w.

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ORIGINAL ARTICLE

Bovine coronavirus in Uruguay: genetic diversity, risk factors and transboundary introductions from neighboring countries

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Abstract

Bovine coronavirus (BCoV) is a recognized cause of severe neonatal calf diarrhea, with a negative impact on animal welfare, leading to economic losses to the livestock industry. Cattle production is one of the most important economic sectors in Uruguay. The aim of this study was to determine the frequency of BCoV infections and their genetic diversity in Uruguayan calves and to describe the evolutionary history of the virus in South America. The overall detection rate of BCoV in Uruguay was 7.8% (64/824): 7.7% (60/782) in dairy cattle and 9.5% (4/42) in beef cattle. The detection rate of BCoV in samples from deceased and live calves was 10.0% (6/60) and 7.6% (58/763), respectively. Interestingly, there was a lower frequency of BCoV detection in calves born to vaccinated dams (3.3%, 8/240) than in calves born to unvaccinated dams (12.2%, 32/263) (OR: 4.02, 95%CI: 1.81–8.90; $p = 0.00026$). The frequency of BCoV detection was higher in colder months (11.8%, 44/373) than in warmer months (1.5%, 3/206) (OR: 9.05, 95%CI: 2.77–29.53, $p = 0.000013$). Uruguayan strains grouped together in two different lineages: one with Argentinean strains and the other with Brazilian strains. Both BCoV lineages were estimated to have entered Uruguay in 2013: one of them from Brazil (95%HPD interval: 2011–2014) and the other from Argentina (95%HPD interval: 2010–2014). The lineages differed by four amino acid changes, and both were divergent from the Mebus reference strain. Surveillance should be maintained to detect possible emerging strains that can clearly diverge at the antigenic level from vaccine strains.

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Rubén Darío Caffarena and María Laura Casaux, Carlos Schild contribute equally to this work.

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Introduction

Bovine coronavirus (BCoV) is recognized as a cause of severe neonatal calf diarrhea (NCD), respiratory tract infections in calves, and winter dysentery in adult cattle [1]. NCD has a negative impact on animal welfare and leads to economic losses to the livestock industry due to the costs of treatment and prophylaxis, increased susceptibility to other diseases, increased mortality, and long-term residual effects, such as reduced growth rates and milk production [2–5]. Neonatal calf diarrhea is the major cause of death in unweaned heifers [6].

Cattle production is one of the main economic sectors in Uruguay, accounting for 33% of the exports and 5% of the gross domestic product, with 11,739,000 head of cattle [7]. Worldwide, Uruguay is one of the main exporters of bovine meat [8] and dairy products [9].

Bovine CoV belongs to the species *Betacoronavirus 1*, which was recently assigned by the International Committee on Taxonomy of Viruses (ICTV) to the order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*,

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subfamily *Orthocoronavirinae,* genus *Betacoronavirus*, and subgenus *Embecovirus* [10]. Members of the species *Betacoronavirus 1* infect not only cattle and wild ruminants [11] but also other mammals such as equids (equine coronavirus) [12], humans (human coronavirus OC43) and pigs (porcine hemagglutinating encephalomyelitis virus) [13].

Bovine CoV has a 32-kb, single-stranded, positive-sense RNA genome – the largest among known RNA viruses [13]. Bovine CoV viral particles are enveloped and pleomorphic and contain five structural proteins. Four are external and glycosylated: the transmembrane (M), the small envelope (E), the hemagglutinin-esterase (HE) and the spike (S) proteins. The other, the nucleocapsid (N) protein, is internal [14, 15].

The biological functions of the S protein of CoV include primary attachment to target cells and membrane fusion. The S protein is cleaved to produce the N-terminal S1 and C-terminal S2 glycopolypeptides. The hypervariable region within S1 is associated with some of the antigenic differences, being the major inducer of virus-neutralizing antibodies, and may also be associated with host range and tissue tropism. Most of the differences in S1 occur between virulent and non-virulent strains [16]. The hypervariable S1 genomic region has been widely used to study the genetic variability and evolution of the virus, including the few studies on molecular characterization of BCoV strains conducted in the South American region [17–19].

To date, little is known about the genetic diversity of BCoV in South America, where the few studies that have been done were mainly restricted to Brazil and Argentina, as mentioned above, and information about its evolutionary history in this region is lacking in the scientific literature. Furthermore, the genetic diversity of BCoV in Uruguay has not been investigated.

The aim of this study was to determine the frequency of BCoV infection in Uruguayan calves, to examine the genetic diversity of the virus, to identify risk factors associated with the frequency of BCoV detection, and to investigate the evolutionary history of BCoV in the South American region through phylogenetic, phylodynamic, and phylogeographic analyses.

Materials and methods

Sample collection and fecal suspensions

A total of 824 samples of feces (763) and intestinal contents (61) were obtained from beef and dairy cattle in Uruguay between July 2015 and December 2017; additional information on sample origins is detailed in Table 1 and Fig. 1. A

Table 1 Sample information

	Diarrhea			Exploitation type		Age (days)						Dams vaccinated[1]		
	Yes	No	ND	Dairy	Beef	1-7	8-14	15-21	22-28	> 28	ND	Yes	No	ND
n	266	297	261	782	42	153	220	113	38	20	280	240	263	321

ND: not determined

[1] Vaccination against neonatal calf diarrhea. Most available vaccines in the Uruguayan market include bovine coronavirus

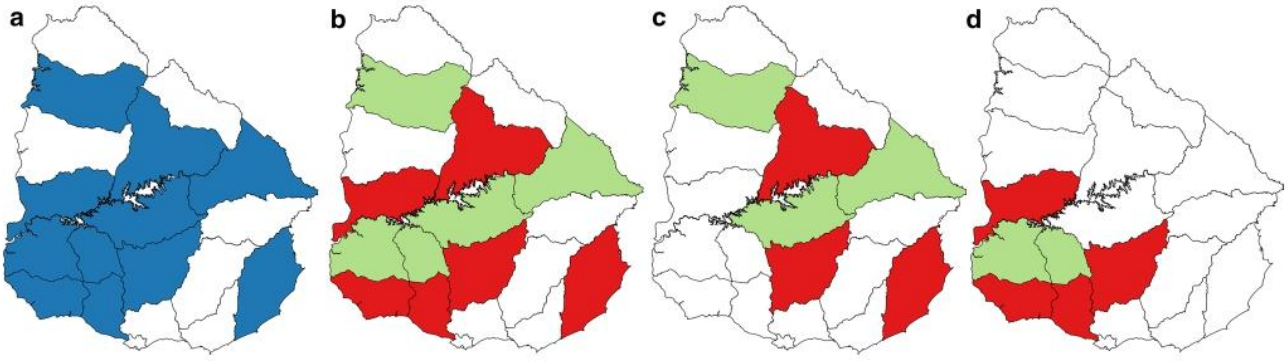

Fig. 1 Map of Uruguay showing the 19 departments into which the country is divided. **a**) Sampling. **b**) Rate of BCoV detection in beef and dairy cattle combined. **c**) Rate of BCoV detection in beef cattle. **d**) Rate of BCoV detection in dairy cattle. Departments from which samples were obtained are shown in blue, those in which BCoV was detected are shown in red, and those in which BCoV was not detected are shown in green

survey conducted to collect information about management and problems associated with dairy cattle farming (the farms were randomly selected and representative of the dairy area in southwestern Uruguay) and the intensive production system facilitated the collection of a large number of samples (782). On the other hand, beef cattle sampling was carried out by personal contact with farmers in order to encompass as many departments (geographic regions) as possible, but the extensive production system of beef cattle in Uruguay hindered access to the samples (42), and this is a limitation of this work. Samples were diluted 1:10 (v:v) in phosphate-buffered saline solution and centrifuged at 3000 *g* for 20 minutes at 4 °C, and supernatants were collected and stored at – 80 °C.

RNA extraction and reverse transcription

Viral RNA was extracted using a QIAamp cador Pathogen Mini Kit (QIAGEN), following the manufacturer's instructions. Reverse transcription (RT) was carried out using RevertAid Reverse Transcriptase (Thermo Fischer Scientific) and random hexamers primers (QIAGEN) following the manufacturer instructions. All RNAs and cDNAs were stored at – 80 °C.

Polymerase chain reaction assay for bovine coronavirus

The initial screening of the samples for identification of BCoV was carried out using a real-time polymerase chain reaction (PCR) targeted the S gene, following a standard operating procedure kindly provided by Dr. Stephanie Rossow from the University of Minnesota Veterinary Diagnostic Laboratory. Briefly, 12.5 µL of SensiFAST™ Probe No-ROX Kit (Bioline), 1.0 µL of 10 µM BCoV Minn F primer (TGTTTTAAAGCTTCCACAAATTTCTG), 1.0 µL of 10 µM BCoV Minn R primer (AACCAGCATCTATAC CAGGACCAT), 0.5 µL of 10 µM BCoV Minn S probe (Cy5-CGTGTAAATTGGATGGGTCTTTGTGTGTAG GT-BHQ-2) and 5.0 µL of nuclease-free water were mixed in 0.2-mL PCR tubes. The PCR cycling conditions were 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 50 °C for 45 seconds.

In order to obtain sequences for evolutionary analysis, positive samples detected by the real-time PCR assay were further amplified using a heminested PCR targeting to the S gene, using primers described elsewhere [17]. Briefly, for the first round of the heminested PCR, 12.5 µL of MangoMix™ (Bioline), 5 µL of cDNA, 3.9 µL of nuclease-free water, 1 µL of dimethyl sulfoxide, 1.3 µL of 10 µM S1NS primer, and 1.3 µL of 10 µM primer S1HA were mixed in 0.2-mL PCR tubes and subjected to an initial step of 5 minutes at 95 °C, followed by 35 cycles of 94 °C for 1 minute, 53.4 °C for 1 minute, and 72 °C for 1 minute, ending with 10 minutes at 72 °C for final extension. For the second round, 12.5 µL of MangoMix™ (Bioline), 2 µL of the PCR product from the first round, 7.5 µL of nuclease-free water, 1 µL of dimethyl sulfoxide, 1 µL of 10 µM S1NS primer, and 1 µL of 10 µM primer S1NAS were mixed in 0.2-mL PCR tubes and subjected to an initial step of 5 minutes at 95 °C, followed by 40 cycles of 94 °C for 1 minute, 58.4 °C for 1 minute and 72 °C for 1 minute, ending with 10 minutes at 72 °C for final extension. The predicted PCR products were 785 bp and 488 bp long for first and second round, respectively (modified from reference 17).

Purification and sequencing of PCR products

PCR products were visualized in 2% agarose gels, and positive samples were purified using a PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) according to the manufacturer's instructions, and both DNA strands were sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were deposited in the GenBank database with accession numbers MK318150-MK318179.

Phylogenetic analysis

Partial spike sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/, Table S1) and, together with the Uruguayan sequences obtained in this study, were aligned using Clustal W, implemented in MEGA 7 software [20]. A curated alignment of 443 nucleotides (nt) was obtained (corresponding to positions 24,984-25,426 of the Mebus strain). The nucleotide substitution model that best fit the alignment (TIM3+I+G_4) was chosen and a maximum-likelihood tree was constructed using W-IQ-TREE (available at http://iqtree.cibiv.univie.ac.at) [21]. The branch support was estimated using the approximate likelihood-ratio test (aLRT) [22].

Phylodynamic and phylogeographic analysis

Considering that South American strains grouped together in two lineages, all of the available sequences of the S region of the BCoV genome available in GenBank from South America were downloaded (Table S2), although some of the Brazilian and Argentinean sequences were shorter than the ones obtained in our study. In order to include these sequences from the South American region, the fragment used for evolutionary analysis was smaller than that used for phylogenetic analysis. Sequences were aligned using Clustal W implemented in MEGA 7 software [20], and an alignment of 332 nt was obtained (corresponding to positions 25,021-25,352 of the Mebus strain). The temporal structure of the dataset was evaluated using TempEst [23]. The

substitution model that best fit the alignment was determined using MEGA 7 software, and used as prior (TN93, [24]) in the analysis implemented in the BEAST v1.8.4 package [25]. Combinations of molecular clocks (strict, relaxed lognormal and relaxed exponential) and coalescent tree priors (constant, exponential, and skyline) were evaluated using Bayes factors. The uncorrelated relaxed with exponential distribution molecular clock and the Bayesian Skyline coalescent model were selected, and the country location was used as a trait for the phylogeographic analysis. The Markov chain Monte Carlo length was 200 million generations, obtaining 10,000 parameters samples. Effective sample size (ESS) was evaluated in Tracer v1.6.0, and ESS values higher than 200 for all parameters were accepted.

A maximum clade credibility tree (MCCT) was obtained using TreeAnnotator software from the BEAST v.1.8.4 package and visualized in FigTree v1.4.3. A Bayesian Skyline plot was generated using Tracer v1.6.0.

Signature patterns distinguishing Uruguayan lineages

Viral Epidemiology Signature Pattern Analysis (VESPA) [26], available at: https://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html) was used to detect patterns that could differentiate the lineages of BCoV circulating in Uruguayan cattle, using only the local strains and the translated alignment obtained for the phylogenetic analysis (the first nucleotide was excluded to have an in-frame translation). Moreover, both lineages were compared with the reference strain Mebus, which is used in the vaccines currently available on the market.

Statistical analysis

Data were organized and graphics were generated using Microsoft Office Excel. Categorical data were evaluated using Pearson's chi-squared test with jamovi software (available at: https://www.jamovi.org/). Differences were considered statistically significant if the p-value was lower than 0.05. Odds ratios (OR) and 95% confident intervals (CI) were calculated for groups with statistically significant differences, using jamovi software. In multiple comparison Chi-square tests, the Bonferroni correction was applied.

Results

Frequency of BCoV detection in Uruguay

The study covered 11 departments of the 19 into which Uruguay is geographically divided (Fig. 1a). Bovine CoV was detected in 6 out of 11 departments (55%) (Fig. 1b); the frequency of detection in each department was as follows: 6.9% (8/116) in Colonia, 7.1% (2/28) in Florida, 14.7% (21/143) in Río Negro, 22.2% (2/9) in Rocha, 6.4% (15/233) in San José, and 12.5% (1/8) in Tacuarembó. BCoV was detected in 3 of the 6 departments sampled for beef cattle (50%) (Fig. 1c), and 4 of the 6 departments sampled for dairy cattle (67%) (Fig. 1d).

The overall detection rate of BCoV in Uruguay was 7.8% (64/824). The frequency of BCoV detection in dairy and beef cattle was 7.7% (60/782) and 9.5% (4/42), respectively (Fig. 2a); this difference was not statistically significant ($p = 0.69$). The detection rate of BCoV in samples from deceased calves (10.0%, 6/60) was higher than in samples from live calves (7.6%, 58/763), although this difference was not statistically significant ($p = 0.50$) (Fig. 2b).

Interestingly, calves born to unvaccinated dams showed higher frequency of BCoV infection (12.2%, 32/263) than calves born to vaccinated dams (3.3%, 8/240). This difference was statistically significant (OR: 4.02, 95%CI: 1.81–8.9; $p = 0.00026$) (Fig. 2c).

As shown in Fig. 2d, BCoV was detected in 6.5% (10/153), 5.5% (12/220), 11.5% (13/113), 18.4% (7/38), and 15.0% (3/20) of the calves in the first, second, third, and fourth week and after the fourth week of life, respectively. A statistically significant difference was observed between the second and the fourth week of age (OR: 3.91, 95%CI: 1.43-10.70; $p = 0.0047$). No statistical differences were observed between BCoV frequency of detection and diarrhea when all age groups were analyzed together: 6.5% (17/263) in diarrheic and 10.8% (32/295) in non-diarrheic calves ($p = 0.068$), however, in the first week of calves' life, the BCoV detection rate was statistical higher in diarrheic 12.1% (7/58) than non-diarrheic 3.2% (3/92) calves (OR: 4.21, 95%CI: 1.04-16.99; $p = 0.030$). In the other age groups, the BCoV frequency was lower in diarrheic versus non-diarrheic calves: 3.9% (6/152) vs. 8.8% (6/68), 2.9% (1/34) vs. 15.2% (12/79), 14.3% (1/7) vs. 19.4% (6/31) and 12.5% (1/8) vs. 16.7% (2/12), in the second, third, and fourth week, and after the fourth week of life, respectively, but no statistically significant differences were observed.

As shown in Fig. 2e, BCoV was detected with a seasonal distribution, mainly in June, July and August, which are the coldest months in Uruguay. In addition, two groups were analyzed based on monthly average temperature using a cutoff value of 13 °C considering that the mean monthly temperature is below the cutoff in June, July and August and above the cutoff in the rest of the months. The frequency of BCoV detection was significantly higher in the months with average temperature <13 °C (11.8%, 44/373) than in those with an average temperature >13 °C (1.5%, 3/206) (OR: 9.05, 95%CI: 2.77-29.53, $p = 0.000013$).

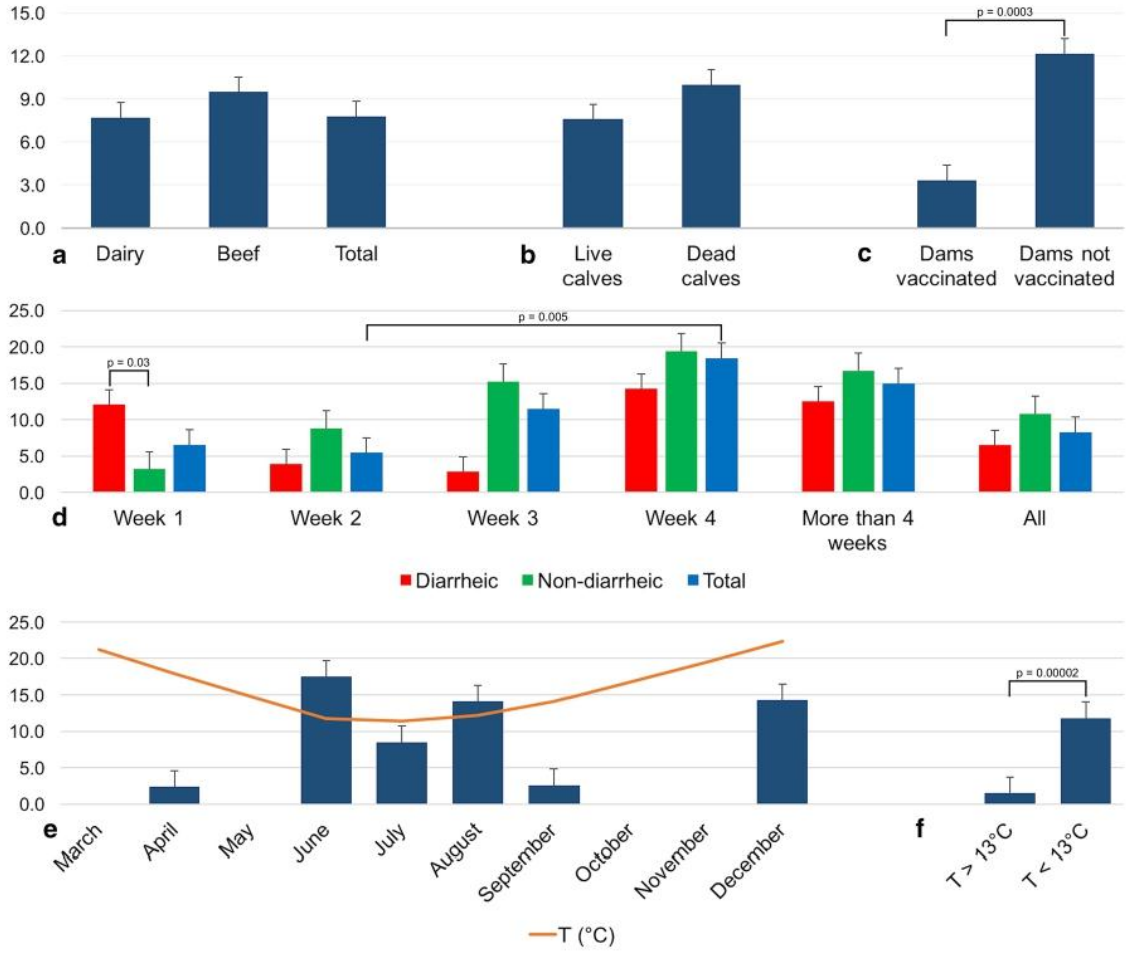

Fig. 2 Comparison of BCoV detection rates in different groups. **a**) Frequency of BCoV detection in beef vs. dairy cattle. **b**) Frequency of BCoV detection in live vs. dead calves. **c**) Frequency of BCoV detection in calves born to vaccinated vs. unvaccinated dams. **d**) Frequency of BCoV detection according to the age in weeks in diarrheic and non-diarrheic calves, and the total number of calves. **e**) Frequency of BCoV detection according to the month of sampling. **f**) Frequency of BCoV detection according to the ambient temperature. Comparisons between groups with statistically significant differences are shown

Phylogenetic analysis

In Fig. 3, the phylogenetic relationship between the Uruguayan BCoV strains and those for which sequences were obtained from the GenBank database is shown. Geographically associated (continent-specific) lineages were observed, and Uruguayan strains grouped in the two South American lineages, one of them with Argentinean strains and the other with Brazilian strains.

Phylodynamic and phylogeographic analysis of BCoV in South America

An MCCT (Fig. 4a) also showed that the Uruguayan BCoV strains grouped together in two different lineages: one with Argentinean and the other with Brazilian strains. The substitution rate estimated in this analysis was 1.39×10^{-3} substitutions/site/year (s/s/y) (95% highest posterior density [95%HPD] interval: 8.3×10^{-4}–2.0×10^{-3}), and the most recent common ancestor (MRCA) of each lineage was dated to 1992 (95%HPD interval: 1985–1994) located in Argentina with a probability of 0.99, and in 2000 (95%HPD interval: 1994–2002) located in Brazil with a probability of 0.99. More information about key nodes is detailed in Table 2.

These BCoV lineages were estimated to have entered Uruguay in 2013: one of them from Brazil (95%HPD interval: 2011–2014) and the other from Argentina (95%HPD interval: 2010–2014) (Fig. 4c).

A Bayesian skyline plot showed that the population size of BCoV in South America was constant in the last two decades, with some minor fluctuations (Fig. 4b).

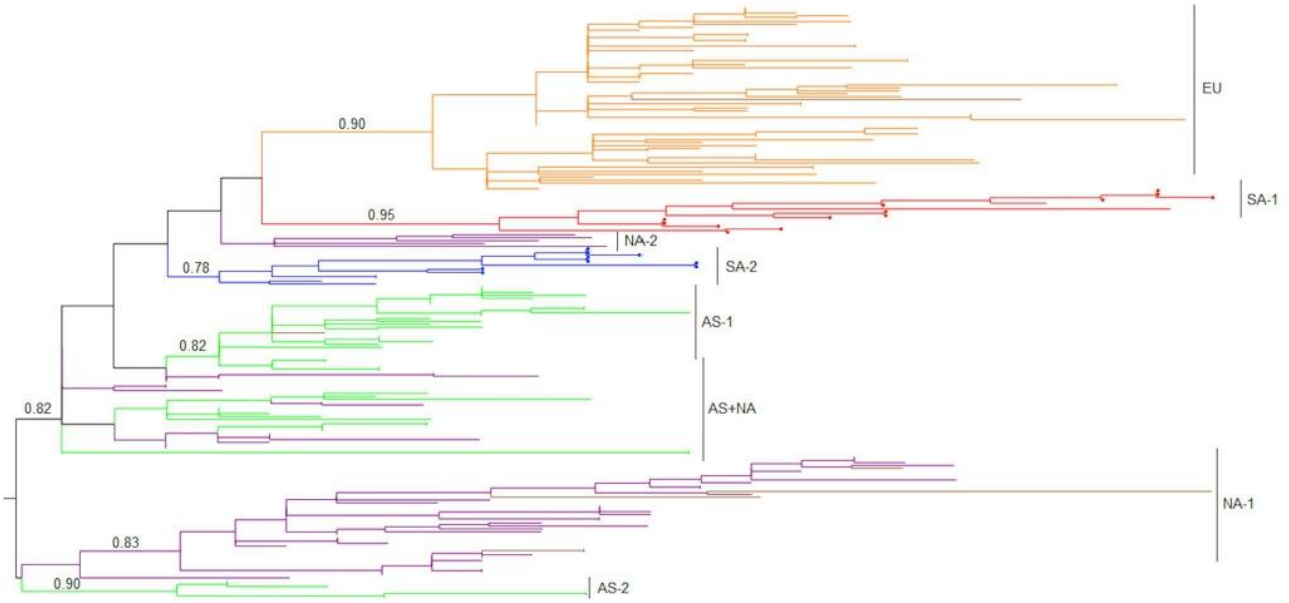

Fig. 3 Phylogenetic analysis. A maximum-likelihood tree was constructed with TIM3 plus gamma plus invariant sites as the nucleotide substitution model. Uruguayan strains from this study are indicated by red and blue circles, and the Mebus strain is indicated by a black circle. aLRT values at key branches and continent-specific lineages are shown. Branches are colored according to the continent of strain isolation: orange (EU), purple (NA), green (AS), red (SA, lineage with Argentinean strains), blue (SA, lineage with Brazilian strains), and brown (strains from a continent of isolation different from the continent-specific lineage). AS, Asia; EU, Europe; NA, North America; SA, South America

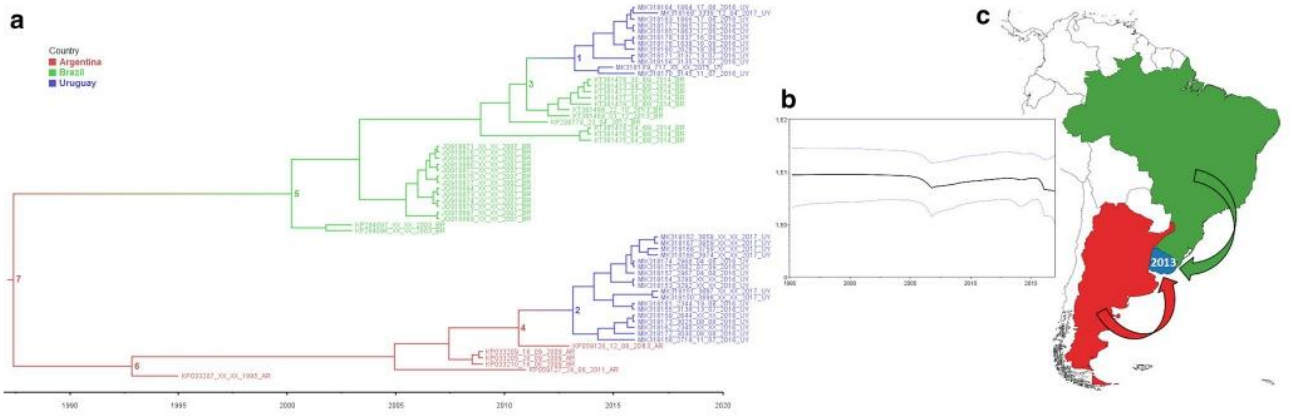

Fig. 4 Phylogeographic analysis. **a**) Maximum clade credibility tree. Branches are colored by most probable country location: blue for Uruguay, green for Brazil, and red for Argentina. Key nodes 1-7 are indicated (for which more information is available in Table 2). **b**) Population dynamics of BCoV in South America. The Bayesian skyline plot shows the evolution in population size. Median (dark line) and upper and lower 95% HPD (blue lines) estimates of effective population size (*y*-axis) through time in years (*x*-axis) are shown. **c**) The two entries of BCoV to Uruguay in 2013 are indicated with arrows: red from Argentina and green from Brazil

Signature patterns distinguishing Uruguayan lineages

Four amino acid (aa) differences between the two BCoV lineages circulating in Uruguay were detected. These changes were N44D, A46S, S62T and Y77H (positions refer to the partial sequences used for the analysis). When the Uruguayan BCoV sequences were compared with the Mebus reference strain, six aa changes (4.0%) were observed in all the Uruguayan strains, and three additional aa changes were observed, totalizing nine aa changes (5.4%), with more than 75% of the Uruguayan sequences containing these changes (Fig. 5).

Table 2 Key nodes for the information shown in Fig. 4

Node	Country	Year	95% HPD year	Country probability
1	Uruguay	2013	2011 - 2014	0.99
2	Uruguay	2013	2010 - 2014	0.99
3	Brazil	2011	2008 - 2012	0.99
4	Argentina	2010	2007 - 2012	0.81
5	Brazil	2000	1994 - 2002	0.99
6	Argentina	1992	1985 - 1994	0.97
7	Argentina	1987	1966 - 1994	0.72

Discussion

NCD can be associated with more than 50% of fatalities in calves [6]. Although multiple factors are involved, BCoV is one of the various pathogens associated with this syndrome. The cattle industry is one of the main sources of income in Uruguay, and this country is one of the main exporters of meat and dairy products worldwide [7–9]. In this work, we demonstrate the circulation of BCoV in calves in Uruguay, with a frequency of 7.8%, which is higher than in Argentina (1.71%) [18] but lower than in Brazil (14.9 - 33.3%) [17, 27, 28].

The frequency of BCoV detection was higher in beef cattle than dairy cattle, in contrast to what has been reported in Argentina [18], although the difference observed in our study was not statistically significant. Moreover, it is worth mentioning that the sampling was not evenly distributed because many more samples were analyzed from dairy calves (782) than from beef calves (42). In addition, dairy cattle are concentrated in the southwest region of the country, whereas beef cattle are dispersed throughout the rest of the country [7]. Despite the difference in sampling, we considered the inclusion of beef cattle samples relevant, but the results should be interpreted with caution. We could not determine if the observed differences were due to the breed of cattle, calf management, production type and/or geographical region, so further studies are needed to clarify these points.

	455	457	458	462	465	470	484	492	494	495	499	501	503	510	514	525	528	531	533	543	544	545	571	578	589
Mebus	T	Q	F	P	V	H	S	D	S	L	N	P	I	S	T	H	A	N	L	S	T	G	Y	T	V
MK318162	I	.	.	.	.	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	W	H	.	.
MK318161	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	.	H	I	.
MK318159	I	.	.	.	.	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	W	H	.	.
MK318175	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318158	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	W	H	.	.
MK318157	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318174	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318173	I	.	.	.	.	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	W	H	.	.
MK318172	I	.	.	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	?	.	W	H	.	.
MK318166	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	.	A	.	.	H	I	F
MK318167	.	.	S	.	A	D	T	N	A	.	S	F	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318168	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318155	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	.	H	I	.
MK318154	.	P	.	.	A	D	T	N	A	.	S	S	.	.	I	Y	V	D	.	A	.	.	H	.	.
MK318153	.	.	.	.	A	D	T	N	A	.	S	S	.	.	I	Y	V	D	.	A	.	.	H	.	.
MK318152	.	.	S	.	A	D	T	N	A	.	S	F	.	.	.	Y	.	D	.	A	.	.	H	I	.
MK318151	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	.	H	I	.
MK318150	.	.	S	.	A	D	T	N	A	.	S	S	.	.	.	Y	.	D	M	A	.	.	H	I	.
MK318178	.	.	S	.	A	D	T	.	.	.	S	S	T	T	.	.	.	D	.	A	K	.	H	I	.
MK318176	.	.	S	.	A	D	T	.	.	.	S	S	T	T	.	.	.	D	.	A	K	.	H	I	.
MK318165	.	.	.	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318164	.	.	.	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318177	.	.	.	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318169	.	.	S	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318156	.	.	S	.	A	D	T	?	?	.	S	S	.	?	.	.	.	D	?	A	.	.	H	I	.
MK318163	.	.	.	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318160	.	.	.	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	K	.	H	I	.
MK318171	.	.	S	H	A	D	T	.	.	V	S	S	.	T	.	.	.	D	.	A	.	.	H	I	.
MK318170	I	.	S	.	A	D	T	.	.	.	S	S	.	T	.	.	.	D	.	.	.	.	H	I	.
MK318179	I	.	S	.	S	D	T	.	.	.	S	S	.	T	.	.	.	D	.	A	.	.	H	I	.

Fig. 5 Amino acid sequence alignment. Comparisons were done between the Mebus strain and the Uruguayan strains, and also between the two lineages circulating in Uruguay. Accession numbers of Uruguayan strains related to Argentinian and Brazilian strains are shown in red and green, respectively. The Mebus strain sequence was used as a reference. Numbers above the amino acid sequence indicate the position of the complete spike sequence of strain Mebus (accession number: U00735). Dots indicate conserved positions, and amino acid changes are represented using the corresponding one-letter symbol. Positions with more than 80% of strains containing differences are indicated by squares. Red, 100% of Uruguayan strains different from Mebus; orange, > 75% of Uruguayan strains different from Mebus; pink, > 50% of Uruguayan strains different from Mebus; dark blue, 100% of strains with differences between lineages; in light blue, > 50% of strains with differences between lineages, black, and other changes

Strategies to prevent NCD should be directed toward enhancing host immunity and reducing the viral load in the environment [29]. There is evidence that the latter goal is not being met in Uruguay [30]; however, vaccination against NCD is a strategy used to enhance host immunity (48% of the calves in dairy farms in this study were born to vaccinated dams, Table 1). Interestingly, we observed that vaccination of the dam was associated with a reduced likelihood of BCoV being detected in the calves. It is important to clarify that the information collected about vaccination in this study refers to vaccination against NCD and not specifically against BCoV, and although most vaccines include a strain of BCoV, there are some exceptions. Therefore, the impact of vaccines on the reduction of BCoV needs to be investigated further. On the other hand, BCoV detection was higher in dead calves with diarrhea than in live calves (although not statistically significant), in concordance with previous data [31].

Interestingly, the frequency of BCoV detection and the proportion of diarrheic to non-diarrheic samples containing the virus varied with the age of the calves. While the frequency of BCoV detection was higher from the third week of age, the higher frequency of BCoV detection in diarrheic calves than in non-diarrheic calves was only observed in the first week of life. Colostral antibodies persist in calves for approximately 3 weeks, and between the third and fourth week of age, antibody titers from passive transfer are low, and the calf is just beginning to mount its own antibody responses to environmental microbiota [32]. The higher frequency of BCoV detection in the third and fourth weeks of age in our study might correspond to this decline in colostral immunity. On the other hand, the only age group with a higher BCoV detection rate in diarrheic samples than in non-diarrheic samples was the first week of age, in agreement with previous studies in which the susceptibility of calves to NCD caused by BCoV was found to be higher in the first days of life [33–36].

Notably, BCoV was detected mainly in the coldest months. Since BCoV is more stable at lower temperatures and lower levels of ultraviolet light [37], this might be a reason why BCoV is most frequently detected in winter [38, 39]. In Uruguay, according to the Uruguayan Institute of Meteorology (INUMET), in June, July and August (in addition to the temperature), the time of direct insolation, the vapor pressure, and the cumulative rainfall are also lower than in the other months, and the average relative humidity and the atmospheric pressure are higher than in the remainder of the months. Therefore, although we focused on the average temperature in this report, some (or all) of the above-mentioned factors could have been involved in the higher detection rate of BCoV in those three months. The higher frequency of BCoV detection in winter may be due to cattle shedding the virus year after year in the winter months, as the incidence of coronavirus shedding in non-vaccinated cows that delivered in the winter months has been reported to increase at parturition [38]. Calves born to BCoV carrier dams have a significantly higher risk of developing BCoV-induced diarrhea due to periparturient exposure from fecal contamination, and if calves are raised in groups, transmission between them is expected [31, 38, 40, 41].

Although only one serotype of BCoV has been identified, there is increasing evidence of divergence of recent isolates from the historical reference strain used for most vaccine formulations (Mebus strain) [18, 42, 43]. In this regard, the strains detected in this study are divergent from the Mebus strain, as observed in the phylogenetic analysis, and nine amino acid differences were observed between the Uruguayan strains and Mebus. Four of these differences (together with several nucleotide changes, not shown), are associated with divergence between the Uruguayan lineages, although both appear to have entered to Uruguay in the same year and from neighboring countries. Surveillance is necessary to determine if vaccine strains are still effective against the new variants that are emerging, and if strains are found to have antigenic differences, they should be studied and possibly included in the vaccine formulations. Uruguayan BCoV strains that diverged from the Mebus strain were found to cluster with the Argentinean isolate Arg95. Mebus-induced neutralizing antibodies were capable of neutralizing the Arg95 strain and vice versa, indicating that the aa differences were not enough to establish a new serotype [18]. It is worth mentioning that some strains (56%, 34/60) could not be amplified by conventional PCR, despite the fact that they were detected in positive samples by real-time PCR, which could be explained by a difference in the sensitivity of the methods and/or possible mutations in the primer regions.

Bayesian MCCT confirmed the two lineages circulating in Uruguay that were identified in the ML tree. One of the lineages entered from Argentina, and the other entered from Brazil, and both lineages entered in the same year (2013), which strongly suggests that there are biosecurity shortcomings leading to the transboundary spread of BCoV between these countries. However, factors that may have led to the introduction of these two viral lineages in the same year from two neighboring countries remain largely unknown. The substitution rate estimated in this analysis was 1.39×10^{-3} s/s/y, faster than previous estimates: 6.1×10^{-4} s/s/y [44] and 8.7×10^{-4} s/s/y [45]. Both previous analyses were carried out using complete S gene sequences (the first together with other betacoronavirus 1), while in our analysis, partial S genome sequences corresponding to a hypervariable region were used, which is expected to show a faster evolutionary rate and could explain this difference. The MRCA of the BCoV lineages circulating in South America was dated to 1987, and both lineages have been circulating for approximately 20 years on this continent: one of them

in Argentina since 1992, and the other in Brazil since 2000, both spreading later to Uruguay. Based on our analyses, since the entry of BCoV lineages into South America, the population size seems to have been constant, although low fluctuations can be observed.

Conclusions

The two lineages of BCoV detected in this study have been circulating in Uruguayan cattle since 2013, on both beef and dairy farms. BCoV-positive calves in their first week of life are more likely to have diarrhea than older BCoV-positive calves. Winter months with lower temperatures were associated with a higher frequency of BCoV detection. Field BCoV strains in Uruguay are not divergent enough from reference vaccine strains to affect their antigenicity, suggesting that vaccination with currently available vaccines could represent an effective BCoV control strategy, although surveillance should be maintained to detect possible emerging strains that could diverge at the antigenic level from vaccine strains. Biosafety capabilities and biosecurity shortcomings between South American countries should be further assessed.

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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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Artículo 3. Análisis del sesgo de uso de codones en todo el genoma de coronavirus bovino

Este artículo permitió ahondar en el conocimiento evolutivo del virus, permitiendo entonces comprender de manera más exhaustiva al mismo. En primer lugar se determinaron los factores que influencian el sesgo en el uso de codones en el virus, siendo el sesgo mutacional, la frecuencia relativa de dinucleótidos y la distribución geográfica los principales. Además, se observó que los codones utilizados por el virus están menos adaptados a su hospedero que los propios genes del hospedero, siendo que el virus utiliza preferentemente aquellos codones que son menos utilizados por los genes de su hospedero.

Este artículo permitió además, obtener el conocimiento para poder aportar información muy valiosa durante la pandemia debido a otro coronavirus, el SARS-CoV-2, documentada en un artículo científico, que se describe más adelante en esta tesis (Un análisis exhaustivo de la composición del genoma y los patrones de uso de codones de coronavirus emergentes).

Estos resultados fueron publicados en la revista Virology Journal: **Castells M**, Victoria M, Colina R, Musto H, Cristina J. Genome-wide analysis of codon usage bias in Bovine Coronavirus. Virol J. 2017;14(1):115. Jun 17. doi:10.1186/s12985-017-0780-y.

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Genome-wide analysis of codon usage bias in Bovine Coronavirus

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Abstract

Background: Bovine coronavirus (BCoV) belong to the genus *Betacoronavirus* of the family *Coronaviridae*. BCoV are widespread around the world and cause enteric or respiratory infections among cattle, leading to important economic losses to the beef and dairy industry worldwide. To study the relation of codon usage among viruses and their hosts is essential to understand host-pathogen interaction, evasion from host's immune system and evolution.

Methods: We performed a comprehensive analysis of codon usage and composition of BCoV.

Results: The global codon usage among BCoV strains is similar. Significant differences of codon preferences in BCoV genes in relation to codon usage of *Bos taurus* host genes were found. Most of the highly frequent codons are U-ending. G + C compositional constraint and dinucleotide composition also plays a role in the overall pattern of BCoV codon usage.

Conclusions: The results of these studies revealed that mutational bias is a leading force shaping codon usage in this virus. Additionally, relative dinucleotide frequencies, geographical distribution, and evolutionary processes also influenced the codon usage pattern.

Keywords: Bovine, Coronavirus, Codon usage, Evolution

Background

Coronaviruses belong to the family *Coronaviridae* and are the largest enveloped single-strand RNA viruses, ranging from 26 to 31 kilobases in genome size [1, 2]. These viruses infect a wide range of avian and mammalian species, and are responsible for enteric or respiratory infections [3]. There is a rising concern about the emergence of two human coronaviruses, Severe acute respiratory syndrome-related coronavirus (SARS-CoV) and Middle-East respiratory syndrome coronavirus (MERS-CoV), who emerged in 2002 and 2012, respectively [4, 5]. Both SARS-CoV and MERS-CoV have a zoonotic origin, revealing the importance of the control of coronaviruses associated with domestic animals in close contact with human populations [6].

Coronaviruses consists of four genera named *Alpha-, Beta-, Gamma- and Deltacoronavirus* based on phylogenetic distance of highly conserved domains. In turn, *Betacoronavirus* genus is divided into four clades, namely A to D. Bovine coronavirus (BCoV) belongs to the *Betacoronavirus* genus clade A [7, 8].

BCoV are widespread around the world and cause enteric or respiratory infections among cattle [9, 10]. These viruses are associated with different syndromes in cattle, ranging from neonatal calf diarrhea, winter dysentery in adult cattle, to respiratory infection in cattle of different age groups [11].

BCoV infection leads to important economic losses to the beef and dairy industry throughout the world, associated with decreased performance, morbidity, mortality, direct cost of treatment of sick animals, and long-term effects on health and productivity of surviving calves [10, 12].

BCoV is closely related to the Human coronavirus OC43 (HCoV-OC43), isolated in 1967 [13]. Recent

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studies revealed that HCoV-OC43 resulted from a zoonotic transmission from bovine to human [6].

The redundancy of the genetic code provides evolution with the opportunity to adjust the efficiency and accuracy of protein production, while preserving the same amino acid sequence [13]. The relation of codon usage among viruses and their hosts may affect viral fitness, evasion from host's immune system and evolution [14–16]. Synonymous triplets are generally not used randomly, and the main forces that drive this bias from equal usage are natural selection and mutational biases [17, 18]. Therefore, the study of codon usage in viruses can reveal important information about virus evolution, regulation of gene expression and protein synthesis [19].

In the present study, we performed comprehensive analyses of codon usage and composition of BCoV strains and explored the possible leading evolutionary determinants of the biases found.

Methods

Sequences

Complete genome sequences for 15 BCoV strains were obtained from GenBank database (available at: http://www.ncbi.nlm.nih.gov). For strain names and accession numbers see Additional file 1. By concatenation of different genome ORF's sequences, different datasets were constructed: one dataset includes the concatenation of nonstructural region ORFs (ORF1ab), a second one containing the structural region ORFs (hemagglutinin-esterase, spike, envelope, membrane, nucleocapsid) and a third including the concatenation of nonstructural and structural ORFs. For each strain the ORFs were aligned using the MUSCLE program [20]. The alignment of concatenation of nonstructural and structural ORFs is available in Additional file 2.

Data analysis

Codon usage, dinucleotide frequencies, base composition, the relative synonymous codon usage (RSCU) [21], the effective number of codons (ENC) [22], aromaticity (Aromo) and hydrophathicity (Gravy) values were calculated using the program CodonW (available at http://sourceforge.net/projects/codonw). The total G + C genomic content and G + C content at first, second and third codon positions were calculated using the Codon W program and EMBOSS Cusp program (available at http://emboss.bioinformatics.nl/cgi-bin/emboss/cusp). The RSCU values of *Bos taurus* cells were obtained from Kazusa database (available at: http://www.kazusa.or.jp/codon/). Codon usage preferences in BCoV in relation to the codon usage of *Bos taurus* were established by means of the use of the codon adaptation index (CAI) [23]. CAI was calculated using the approach of Puigbo et al. [24]. This method permits to compare a given codon usage (BCoV) to a predefined reference set (*Bos taurus*). In order to show whether the BCoV genes are well adapted to the codon usage of the reference set, as measured by CAI, we constructed a dataset composed of 22 *Bos taurus* genes selected at random and obtained from ARSA at DNA Database of Japan (available at: http:/http://www.ddbj.nig.ac.jp/arsa). The RCSU values of those 22 *Bos taurus* genes were found to be similar to the RCSU values obtained from the Kazusa database (see Additional file 3). Moreover, a strong positive correlation are found among the RSCU of those genes and the RSCU obtained from Kazusa database by Spearmans'rank correlation test [25] ($r = 0.956$, $P < 0.00001$).

Statistically significant difference among CAI values was determined by applying a Wilcoxom & Mann-Whitney test [25]. To discern if the statistically significant differences in the CAI values arise from codon preferences, we used e-CAI [26] to calculate the expected value of CAI (eCAI) at the 95% confident interval. A Kolmogorov-Smirnov test for the expected CAI was also performed [26].

Multivariate analysis

Correspondence analysis (COA) is a type of multivariate analysis that permits a geometrical representation of the sets of rows and columns in a dataset [27, 28]. Each ORF is represented as a 59-dimensional vector and each dimension corresponds to the RSCU value of each codon (excluding AUG, UGG and stop triplets). Major trends within a dataset can be established using measures of relative inertia and genes ordered according to their position along the different axes [29]. COA was performed on the RSCU values by means of the use of the CodonW program. Correlation analysis was performed using Spearman's rank correlation analysis method [25].

Phylogenetic analysis

In order to gain insight into the genetic variability and evolution of BCoV, a phylogenetic tree analysis was performed for all BCoV strains enrolled in these studies, using complete genome codes. Sequences were aligned using the MUSCLE program [20]. Once aligned, the FindModel program (available at: https://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to identify the optimal evolutionary model that best fitted our sequence dataset.

Akaike information criteria (AIC) and the log of the likelihood (logL) indicated that the GTR+ Γ model was the most accurate (AIC = 60,864.73, logL = −30,423.36). Using this model, maximum likelihood trees were

constructed using software from the MEGA 6 program [30]. As a measure of the robustness of each node, we employed the bootstrap method (500 replicas).

Results

General codon usage pattern in BCoV

In order to gain insight into the degree of codon usage bias in BCoV, the ENC's values were calculated for the complete genome of all BCoV strains. A mean value of 43.78 ± 0.07 was obtained for BCoV strains included in these studies. Then, a plot of ENC versus GC3S (ENC plotted against G + C content at the third codon position) was constructed. An ENC-GC3S plot of genes whose codon choice is constrained only by a GC3 mutational bias, will lie on or just below the continuous curve of the predicted ENC values [31]. As shown in Fig. 1, all points lie together under the expected ENC curve, indicating that G + C compositional constraints might play a role in BCoV codon usage. Additionally, a correlation analysis between ENC and GC3S showed significant results ($r = 0.811$, $p = 0.004$).

The aromaticity (Aroma) and hydropathicity (Gravy) values of a given gene product can be indicative of the effect of translation or natural selection [32]. For these reasons, the effect of natural selection on BCoV codon usage was studied by correlation analysis between Gravy and Aroma values and nucleotide compositions at the third codon position and ENC values. No significant correlations between Gravy/Aroma values and nucleotide compositions at third codon position as well as between Gravy/Aroma and ENC values were observed (see Additional file 4).

These results indicate that codon bias in BCoV is related to mutational bias, whereas translational control, may be weak or absent in the reading frames of these viruses.

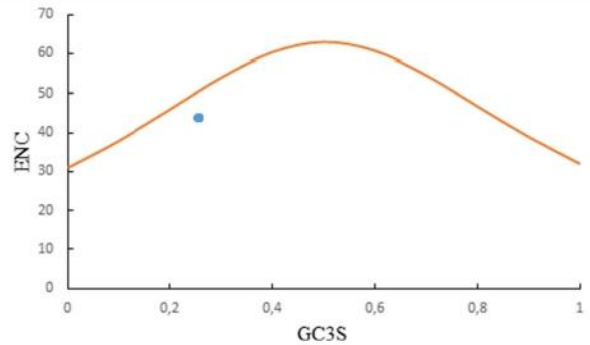

Fig. 1 Effective number of codons (ENC) used in BCoV ORFs plotted against the GC3S The orange curve plots the relationship between GC3S and ENC in absence of selection. *Blue dots* show the results obtained for BCoV strains. Note that the values obtained for ENC and GC3S of all 15 BCoVs enrolled in these studies are very similar (SD of ±0.07 and ±0.0009, respectively) and for that reason they resemble a *single dot* in the figure

Trends in codon usage variation across BCoV strains

To study the trends in codon usage variation among different BCoV genomes, we performed a COA analysis. COA was performed on the RSCU values for the ORF1ab of each BCoV strain enrolled in these studies and we examined the distribution of the strains in the plane defined by the first two principal axes of COA. The first axis generated by the analysis accounts for 43.37% of the total variation, while the second axis accounts for 18.96%. The results of these studies are shown in Additional file 5: Figure S1A. Interestingly, different BCoV isolates are located at different positions in the plane, suggesting that trends in variation of BCoV can be observed. Moreover, these trends correlate with the genetic variability observed by phylogenetic analysis (see Additional file 5: Figure S1B).

Codon usage preferences in BCoV

To compare the codon usage preferences of BCoV with those of *Bos taurus*, the RSCU values of the codons in nonstructural, structural and complete BCoV genome ORFs were calculated and compared with those of *B. taurus*. The results of these studies are shown in Table 1.

The frequencies of codon usage in BCoV ORFs are significantly different in relation to *B. taurus* ones. Indeed, highly biased frequencies were found for UUU (Phe), UUA (Leu), UUG (Leu), CUU (Leu), AUU (Ile), GUU (Val), UAU (Tyr), CAU (His), CAA (Gln), AAU (Asn), GAU (Asp), UCU (Ser), CCU (Pro), ACU (Thr), GCU (Ala), UGU (Cys), CGU (Arg), AGU (Ser) and GGU (Gly). As can be seen, most of the highly preferred codons are U-ending and UpU containing codons, which strongly suggests that mutational bias is a main force shaping codon usage in BCoV (see Table 1). Moreover, most of the highly decreased codons frequencies with respect to *B. taurus* cells are C-ending codons, also suggesting a strong mutational bias in the use of these codons (Table 1). A Wilcoxom & Mann-Whitney test on the frequencies of BCoV codon usage among nonstructural and structural genome regions revealed no significant differences among both regions ($T = 1983$, p-value = 0.758).

Codon usage adaptation in BCoV

In this study, a CAI metric was used as a measure of relative adaptedness of BCoV codon usage to *Bos taurus* host. CAI values for all triplets were calculated for the complete genome ORFs of BCoV strains enrolled in these studies, using *B. taurus* codon usage as the reference set. The results of these studies are shown in Table 2.

Table 1 Codon usage in BCoV, displayed as RSCU[a] values

AA	Cod	BT	NS	ST	Full	AA	Cod	BT	NS	ST	Full
Phe	*UUU*	0.84	1.78	1.68	1.76	Ser	*UCU*	1.03	2.00	1.92	1.98
	UUC	1.15	0.22	0.32	0.24		**UCC**	1.37	0.37	0.50	0.41
Leu	*UUA*	0.37	1.48	1.39	1.46		UCA	0.78	0.84	0.90	0.86
	UUG	0.71	2.09	1.62	1.98		UCG	0.39	0.14	0.26	0.18
	CUU	0.70	1.46	1.48	1.46	Pro	*CCU*	1.07	2.18	1.68	2.01
	CUC	1.25	0.22	0.47	0.28		**CCC**	1.39	0.38	0.82	0.53
	CUA	0.36	0.36	0.57	0.41		CCA	0.99	1.23	1.19	1.22
	CUG	2.58	0.39	0.48	0.42		CCG	0.53	0.21	0.31	0.24
Ile	*AUU*	0.98	1.78	1.66	1.74	Thr	*ACU*	0.88	1.91	2.21	2.00
	AUC	1.56	0.27	0.37	0.30		**ACC**	1.55	0.51	0.63	0.54
	AUA	0.45	0.95	0.97	0.96		ACA	1.00	1.32	0.93	1.20
Met	AUG	1.00	1.00	1.00	1.00		ACG	0.55	0.26	0.24	0.25
Val	*GUU*	0.64	2.24	2.20	2.23	Ala	*GCU*	1.00	2.12	2.07	2.11
	GUC	1.00	0.28	0.44	0.32		**GCC**	1.71	0.57	0.54	0.56
	GUA	0.39	0.63	0.75	0.65		GCA	0.80	1.13	1.12	1.13
	GUG	1.95	0.85	0.62	0.80		**GCG**	0.48	0.18	0.27	0.20
Tyr	*UAU*	0.78	1.63	1.57	1.61	Cys	*UGU*	0.83	1.55	1.54	1.55
	UAC	1.21	0.37	0.43	0.39		**UGC**	1.13	0.45	0.46	0.45
TER	UAA	**	**	**	**	TER	UGA	**	**	**	**
	UAG	**	**	**	**	Trp	UGG	1.00	1.00	1.00	1.00
His	*CAU*	0.75	1.56	1.56	1.56	Arg	*CGU*	0.48	2.17	1.56	2.01
	CAC	1.24	0.44	0.44	0.44		**CGC**	1.17	0.79	0.47	0.70
Gln	*CAA*	0.46	1.02	1.16	1.06		CGA	0.67	0.44	0.39	0.43
	CAG	1.53	0.98	0.84	0.94		**CGG**	1.32	0.32	0.27	0.31
Asn	*AAU*	0.81	1.65	1.70	1.67	Ser	*AGU*	0.87	2.09	1.71	1.98
	AAC	1.18	0.35	0.30	0.33		**AGC**	1.53	0.56	0.71	0.61
Lys	AAA	0.78	1.02	0.93	1.00	Arg	AGA	1.13	1.68	2.03	1.77
	AAG	1.21	0.98	1.07	1.00		AGG	1.20	0.59	1.29	0.78
Asp	*GAU*	0.84	1.70	1.40	1.64	Gly	*GGU*	0.63	2.50	2.46	2.49
	GAC	1.15	0.30	0.60	0.36		**GGC**	1.43	0.58	0.60	0.59
Glu	GAA	0.78	1.17	1.09	1.16		GGA	0.95	0.64	0.63	0.64
	GAG	1.21	0.83	0.91	0.84		GGG	0.98	0.28	0.30	0.28

[a]*RSCU* relative synonymous codon usage, *AA* amino acid, *Cod* codons, *BT Bos taurus* cells, *NS* non structural genome region, *ST* structural genome region, *Full* complete genome coding regions. **, termination codons. Highly increased codons with respect to *B. taurus* cells ($\Delta \geq 0.30$) are shown in italics. Highly decreased codons with respect to *B. taurus* cells are shown in bold

A mean value of 0.638 was obtained for BCoV genes in relation to *B. taurus*; while a mean CAI value of 0.756 was obtained for a *Bos taurus* sequence dataset in relation to the same reference set (see Table 2). In order to evaluate if the differences were statistically significant, we performed a Wilcoxon & Mann-Whitney test. The results of this test revealed that the differences in CAI values are statistically significant ($T = 0$, p-value <0.001). The CAI value obtained for *Bos taurus* genes is higher than the one obtained for BCoV in relation to *Bos taurus* codon usage (see Table 2). This reveals that BCoV genes are relatively less adapted to *Bos taurus* than *Bos taurus* genes itselves.

In order to discern if the statistically significant differences in CAI values arise from codon preferences [24], the expected CAI (e-CAI) values were calculated for BCoV complete genome ORFs sequences in relation to *B. taurus* codon usage reference set. The e-CAI algorithm [26] generated 500 random sequences with the same nucleotide and amino acid composition as the sequences of interest (in this case BCoV sequences). Then, we calculated the CAI values for all of them, and a Kolmogorov-Smirnov test for the e-CAI of these random sequences was performed in order to show if the generated sequences follow a normal distribution. The results of these studies revealed an e-CAI value of 0.656. Kolmogorov-Smirnov test revealed a normal distribution of the generated sequences (Kolmogorov-Smirnov test of e-CAI value of 0.028, which is below the critical value of 0.061). To avoid the effect of extreme compositional constraint and to make sure that CAI is directly correlated with codon usage preferences, Puigbo et al. [26] suggested that if the eCAI value of a gene is higher than its CAI value, it may be considered as evidence of codon usage adaptation. For all BCoV strains, CAI values were found to be lower than their corresponding eCAI values, when compared against *Bos taurus* (eCAI = 0.656, $p < 0.05$).

Taking all these results together, our studies revealed that the CAI values for BCoV genes are different from the CAI values obtained for *B. taurus* sequences and these differences are related to codon usage preferences.

It has been previously shown that dinucleotide biases can play a role in codon usage bias [29]. In order to determine if this is the case in BCoV, the relative abundances of the 16 dinucleotides in BCoV complete genome ORFs were established. The results of these studies are shown in Table 3.

Table 2 Codon adaptation of BCoV genes in relation to *Bos taurus* codon usage, displayed as CAI[a] values

	CAI-BT	%GC	%GC(1)	%GC(2)	%GC(3)
BCoV genes	0.638 ± 0.002	37.09 ± 0.000	45.86 ± 0.000	37.10 ± 0.000	28.32 ± 0.000
Bos taurus genes	0.756 ± 0.048	51.20 ± 6.803	52.17 ± 5.430	38.39 ± 5.546	63.04 ± 12.957

[a]*CAI* codon adaptation index, *CAI-BT* codon adaptation index in relation to Bos taurus reference codon usage set. %GC, percentage of G + C genomic content, %GC(1) through (3), percentage of G + C genomic content at codon positions 1 through 3, respectively. In all cases, mean ± standard deviation values are shown

Table 3 Relative abundance of dinucleotides in BCoV strains and summary of COA

		UU	UC	UA	UG	CU	CC	CA	CG
Mean (S.D.[a])		2.02(±0.001)	0.60(<10^{-3})	1.44(=0)	1.61(<10^{-3})	0.92(<10^{-3})	0.44(±0.001)	0.82(±0.001)	0.25(<10^{-3})
Axis 1[b]	*r*	0.634615	0.163462	0.502747	0.574176	0.181319	−0.354396	0.717033	0.502747
	P	<0.05	0.568	0.080	<0.05	0.528	0.218	<0.05	0.080
		AU	AC	AA	AG	GU	GC	GA	GG
Mean (S.D.[a])		1.47(=0)	0.70(=0)	1.24(<10^{3})	0.93(±0.001)	1.27(<10^{-3})	0.70(<10^{-3})	0.84(±0.001)	0.68(=0)
Axis 1[b]	*r*	0.502747	0.502747	0.502747	0.734890	0.431319	0.199176	0.288462	0.502747
	P	0.080	0.080	0.080	<0.05	0.133	0.490	0.317	0.080

[a]Mean values of BCoV strains relative dinucleotide ratios ± standard deviation. [b]Correlation analysis between the first axis in COA and the sixteen dinucleotides frequencies in BCoV genes is shown

As can be seen, the relative abundance of UpU, UpA and ApU showed a strong deviation from the expected frequencies (i.e. 1.0) (a mean of 2.02, 1.44 and 1.47, respectively), while UpC, CpC, ApC and GpC frequencies were markedly underrepresented (a mean of 0.60, 0.44, 0.70 and 0.70, respectively). The relative abundance of CpG and GpC also showed a strong deviation from the expected frequencies (i.e. 1.0) (a mean of 0.25 and 0.70, respectively).

Discussion

In these studies, we first study the general codon usage pattern in BCoV. When the ENC's values were calculated for the complete genome of all BCoV strains, a mean value of 43.78 ± 0.07 was obtained. Since the ENC values obtained are >40, these results suggest a relatively conserved codon usage bias among different BCoV genomes. This is in agreement with previous reports in other members of the family, like SARS-CoV (mean ENC = 48.99) [33], the avian coronavirus Infectious bronchitis virus (ENC = 42.79) [34] or the Porcine epidemic diarrhea virus (ENC = 47.91) [35]. The ENC-GC3S plot revealed that all values obtained for BCoV lie bellow the continuous curve of predicted ENC values in absence of selection, revealing that G + C compositional constrain play a role in BCoV codon usage (Fig. 1).

Moreover, no significant correlations between both Gravy and Aroma values with nucleotide composition at the third codon position, as well as ENC values (see Additional file 3). This indicates that the role of translational selection in BCoV codon usage bias is weak or absent. Taking all together, the results of these studies suggests that mutational bias is a main force shaping codon usage in this virus.

Then, in order to study the trends in codon usage variation across BCoV strains, a COA analysis was performed on the RSCU values for the ORF1ab of each BCoV strain enrolled in these studies. Different BCoV isolates are located at different positions in the plane defined by the first two principal axes of COA, revealing that trends in codon usage variation can be observed (see Additional file 5: Figure S1A). This is also in agreement with the results found in the phylogenetic analysis of BCoV strains enrolled in these studies (see Additional file 5: Figure S1B) and with recent studies on genetic variability of coronaviruses, showing that BCoVs strains are distributed on three main sub-clusters named C1, C2, and C3 [6]. Sub-cluster C1 includes BCoVs from America and Asia, sub-cluster C2 includes BCoVs from Europe and sub-cluster C3 includes prototype, vaccine, or attenuated BCoV strains [6]. As can be seen in Additional file 5: Figure S1A, BCoV strains Mebus and Quebed, who belong to C3 cluster, are situated in different positions in the plane defined by the first two axes of COA in relation to C1 strains. Moreover, different genetic lineages of C1 sub-cluster are also located at different positions in the plane (Additional file 5: Figure S1A). This is in agreement with the results found in the phylogenetic analysis of BCoVs enrolled in these studies (Additional file 5: Figure S1B). Moreover, BCoV strain BCV-AKS-01, who shows a more distant genetic relation with C3 and C1 BCoVs enrolled in the studies (Additional file 5: Figure S1B), is situated in a different position in the plane defined by the first two axes of COA (Additional file 5: Figure S1A). The results of these studies revealed that evolutionary processes also influenced the codon usage pattern of BCoV.

In these studies, significant differences in codon usage frequencies were found among BCoV and *B. taurus* ones (Table 1). Interestingly, recent studies on human coronaviruses revealed significant biases in nucleotide composition [36]. While the A/G bias is a relatively stable property among coronaviruses, the C/U bias differs significantly in each virus type, with U-counts ranging from 30.7% (SARS-CoV) to 40.3% (HCoV-HKU) and C-counts from 20.3% (MERS-CoV) to 12.9% (HCoV-HKU) [36]. The U-count for BCoV revealed a value of 35.6%, while the C-count shows a value of 15.1%. This is in agreement with the results found in this work and may help to explain the bias

found in BCoV genome composition and codon usage. Moreover, the CAI values for BCoV genes resulted to be significantly different from the ones obtained for *B. taurus*, revealing differences in codon usage preferences (Table 2).

The results of these studies revealed that the relative abundance of CpG and GpC dinucleotides showed a strong deviation from the expected frequencies (Table 3). The under-representation of CpG might be due to its immunostimulatory properties as recognition of unmethylated CpG by Toll like receptor 9 (TLR9), which leads to activation of several immune response pathways in the host [37]. Moreover, an increase in CpG dinucleotide frequency has been shown to lead to attenuation of replication in RNA viruses [38]. Cytosine deamination and selection against CpG motifs have been proposed as two independent selection forces that shape codon usage bias in coronaviruses [39], suggesting that immune selection may play a role in the observed BCoV codon usage bias. This is in agreement with the results of this work and indicates that the composition of dinucleotides also determines the variation in synonymous codon usage among BCoV.

Conclusions

The results of these studies revealed significant differences in codon preferences in BCoV genes in relation to *B. taurus* codon usage. The overall codon usage among BCoV strains is similar. All U- ending codons are highly frequent codons, which strongly suggests that mutational bias is a leading force shaping codon usage in this virus. G + C compositional constraint influences the codon usage of BCoV. Dinucleotide composition also plays a role in the overall pattern of BCoV codon usage.

Additional files

Additional file 1: Origins of the BCoV strains. A table showing strain names and accession numbers. (DOCX 11 kb)

Additional file 2: Alignment of concatenation of nonstructural and structural ORF's of Bovine coronaviruses. A fasta file originated using the MUSCLE program [20]. (FAS 378 kb)

Additional file 3: Comparison of *Bos taurus* RSCU obtained using Kazusa databasea and the RSCU of *Bos taurus* genes dataset used in these studies. (DOCX 14 kb)

Additional file 4: The correlation between Gravy and Aroma and nucleotide at the third codon position of each codon and ENC values. (DOCX 12 kb)

Additional file 5: Figure S1. In (A) Positions of the BCoV strains in the plot of the first two major axes by correspondence analysis (COA) of relative synonymous codon usage (RSCU) values. In (B) Maximum likelihood phylogenetic analysis of BCoV complete codes. (DOCX 67 kb)

Abbreviations

AIC: Akaike information criteria; Aroma: Aromaticity; *B. taurus*: *Bos taurus*; BCoV: Bovine Coronavirus; CAI: Codon adaptation index; COA: Correspondence analysis; eCAI: Expected value of CAI; ENC: Effective number of codons; Gravy: Hydrophathicity; GTR+ Γ: General Time Reversible plus gamma model; HCoV: Human Coronavirus; logL: The log of the likelihood; ORF: Open reading frame; RSCU: Relative synonymous codon usage; SARS-CoV: Severe Acute Respiratory Syndrome-related coronavirus (SARS-CoV) and Middle-East Respiratory Syndrome coronavirus (MERS-CoV)

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Availability of data and materials

The datasets generated and analyzed during the current study are available in Additional file 2.

Authors' contributions

JC conceived of the study, and participated in its design and coordination. MC have made substantial contributions to the acquisition of data and analysis. MV, RC and HM have been involved in the analysis and in interpretation of the results found in this work, as well as in revising the manuscript critically for important intellectual content. JC wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Ethics approval and consent to participate

Not applicable.

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B.3. Estudio de rotavirus en muestras clínicas

Artículo 4. Análisis filogenéticos de RVA bovino en Uruguay revelan la circulación de genotipos comunes y no comunes, y sugieren transmisión interespecie

Este artículo estuvo enfocado principalmente en comprender este virus a nivel de nuestro país, determinando la frecuencia en la cual circula, estudiando diferentes factores de riesgo, su diversidad genética mediante análisis filogenéticos que permitieron determinar la circulación de genotipos comunes, genotipos raramente detectados a nivel mundial, y además evidenciar reordenamientos genéticos, los cuales además indican que es un virus con capacidad de saltar de especie, incluso con posibilidad de zoonosis.

En cuanto a los principales resultados obtenidos, RVA fue detectado en el 57% (475/833) de las muestras analizadas; 60% en terneros lecheros y 28% en terneros de carne. Se observó una frecuencia de detección de RVA significativamente mayor en terneros con diarrea que sin diarrea (OR: 1,73; $p<0,005$). Además, la carga viral fue significativamente más elevada en terneros con diarrea que sin diarrea ($p=0,007$). Por otro lado, no se observó diferencia entre terneros nacidos de madres vacunadas que de madres no vacunadas. Los genotipos detectados fueron: G6P[11], G6P[5], G10P[11], y G24P[33]. Además, 10 cepas fueron caracterizadas también para los genes VP6 y NSP1-5, obteniendo resultados sumamente interesantes, con evidencia de reordenamientos génicos, algunos de ellos debido a transmisión interespecie, incluida la posibilidad de enventos zoonóticos.

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Phylogenetic Analyses of Rotavirus A from Cattle in Uruguay Reveal the Circulation of Common and Uncommon Genotypes and Suggest Interspecies Transmission

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Abstract: Uruguay is one of the main exporters of beef and dairy products, and cattle production is one of the main economic sectors in this country. Rotavirus A (RVA) is the main pathogen associated with neonatal calf diarrhea (NCD), a syndrome that leads to significant economic losses to the livestock industry. The aims of this study are to determine the frequency of RVA infections, and to analyze the genetic diversity of RVA strains in calves in Uruguay. A total of 833 samples from dairy and beef calves were analyzed through RT-qPCR and sequencing. RVA was detected in 57.0% of the samples. The frequency of detection was significantly higher in dairy (59.5%) than beef (28.4%) calves ($p < 0.001$), while it did not differ significantly among calves born in herds that were vaccinated (64.0%) or not vaccinated (66.7%) against NCD. The frequency of RVA detection and the viral load were significantly higher in samples from diarrheic (72.1%, 7.99 $\log_{10}$ genome copies/mL of feces) than non-diarrheic (59.9%, 7.35 $\log_{10}$ genome copies/mL of feces) calves ($p < 0.005$ and $p = 0.007$, respectively). The observed G-types (VP7) were G6 (77.6%), G10 (20.7%), and G24 (1.7%), while the P-types were P[5] (28.4%), P[11] (70.7%), and P[33] (0.9%). The G-type and P-type combinations were G6P[11] (40.4%), G6P[5] (38.6%), G10P[11] (19.3%), and the uncommon genotype G24P[33] (1.8%). VP6 and NSP1-5 genotyping were performed to better characterize some strains. The phylogenetic analyses suggested interspecies transmission, including transmission between animals and humans.

Keywords: rotavirus; bovine; genotypes; interspecies transmission; diarrhea

1. Introduction

Neonatal calf diarrhea (NCD) is a syndrome of worldwide distribution and the major cause of mortality of dairy calves before weaning [1]. NCD has a negative impact on animal welfare and leads to significant economic losses to the livestock industry [2–5].

Rotavirus A (RVA) is the main pathogen associated with NCD [6,7]. RVA (species *Rotavirus A*; genus *Rotavirus*; subfamily *Sedoreovirinae*; family *Reoviridae*) is a nonenveloped virus with a triple-layered capsid and a genome composed of 11 segments of double-stranded RNA [8]. RVA is widespread in dairy farms in Uruguay, and viable viral particles have been detected in sources of drinking water used for calves [9], suggesting water contamination and waterborne transmission.

Rotaviruses are classified by a binary system of G and P types for VP7 and VP4, respectively, determined by sequence analyses. In 2008, a complete genome classification system, named genotype constellation, assigning a specific genotype to each of the 11 genome segments was developed [10]. The VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of rotavirus strains are classified using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (where x is the genotype number), respectively.

Recently, since the inclusion of gene segments other than VP7 and VP4 in molecular analyses, gene reassortment has been described as a common event in RVA, sometimes between virus strains originated from different hosts, suggesting interspecies transmission [10–13].

Surveys describing the epidemiology of RVA in cattle in South America are mainly restricted to Brazil and Argentina; no published data about RVA epidemiology in Uruguayan calves are available. However, other viruses such as bovine coronavirus and bovine astrovirus have been detected in Uruguay [14,15].

Uruguay is one of the main exporters of beef [16] and dairy products [17]. Furthermore, cattle production is one of the main economic sectors in this country, with almost 12 million head of cattle accounting for 33% of the total exports [18]. The aims of this study are to determine the frequency of RVA infections and to analyze the genetic diversity of the RVA strains detected in Uruguayan calves.

2. Results

2.1. Detection Frequency of RVA in Uruguayan Calves

Rotavirus A was detected in 57.0% (475/833) of the analyzed samples. The frequency of detection was significantly higher in dairy (59.5%, 456/766) than beef (28.4%, 19/67) calves (OR: 3.72, 95% CI: 2.14–6.44; $p < 0.000001$; Figure 1a). The frequency of RVA detection in live calves was higher (58.0%, 444/766) than in deceased calves (46.3%, 31/67), although this difference was not statistically significant ($p = 0.06$; Figure 1b). The frequency of detection in dairy calves born in herds that vaccinated (64.0%, 144/225) or did not vaccinate dams (66.7%, 164/246) against NCD did not differ significantly ($p = 0.5$; Figure 1c). The frequency of RVA detection was significantly higher in samples from diarrheic (72.1%, 173/240) than non-diarrheic (59.9%, 163/272) dairy calves (OR: 1.73, 95% CI: 1.19–2.50; $p < 0.005$; Figure 1d). No seasonal distribution was observed in RVA detection (data not shown).

Rotavirus A was detected in 58.8% (87/148), 70.6% (142/201), 68.2% (75/110), and 52.9% (18/34) of dairy calves in the first, second, third, and fourth weeks of life, respectively (Table 1). Statistically significant differences were observed between the second and the first weeks of age (OR: 1.69, 95% CI: 1.08–2.64; $p = 0.02$), and between the second and the fourth weeks of age (OR: 2.14, 95% CI: 1.02–4.48; $p = 0.04$). The mean age in days of RVA-positive dairy calves was significantly lower in diarrheic than nondiarrheic calves ($p = 0.02$; Table 1).

The RVA viral load was significantly higher in diarrheic than nondiarrheic dairy calves ($p = 0.007$; Table 1), ranging between 1.14×10^4 and 7.36×10^{12} genome copies/milliliter (gc/mL) of feces. In all four age groups, the frequency of RVA detection was higher in diarrheic than nondiarrheic dairy calves: 69.0% (40/58) vs. 52.2% (47/90) in the first week, 72.1% (98/136) vs. 67.7% (44/65) in the second week, 68.8% (22/32) vs. 67.9% (53/78) in the third week, and 85.7% (6/7) vs. 44.4% (12/27) in the fourth week

of age. A statistically significant difference was observed only within the first week (OR: 2.03, 95% CI: 1.01–4.07; $p = 0.04$).

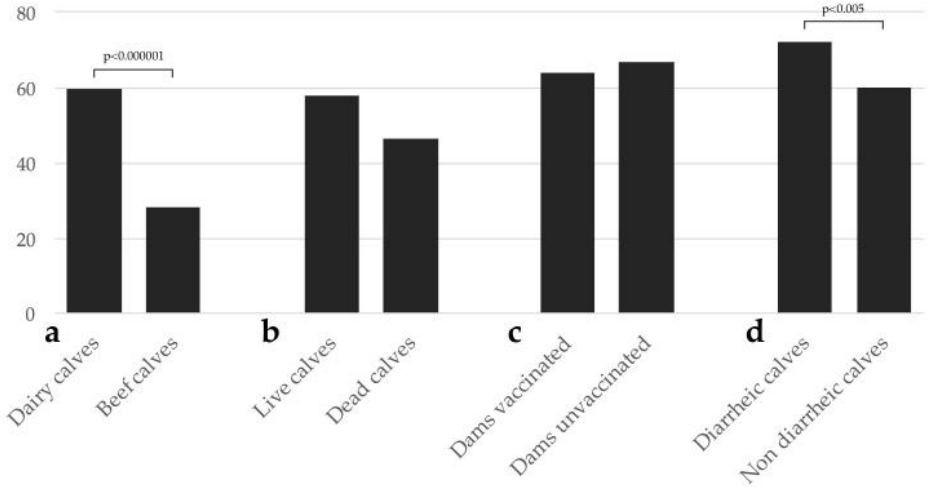

Figure 1. Frequency of Rotavirus A (RVA) detection in calves. (**a**) Frequency of RVA detection in dairy vs. beef calves; (**b**) frequency of RVA detection in live vs. deceased calves; (**c**) frequency of RVA detection in calves from vaccinated [a] vs. unvaccinated dairy herds; (**d**) frequency of RVA detection in diarrheic vs. non diarrheic dairy calves. Comparisons with statistically significant differences are indicated. [a] Most of the vaccines against neonatal calf diarrhea available in Uruguay include two RVA strains.

Table 1. Frequency of RVA detection and viral load in feces of diarrheic and nondiarrheic calves.

			Calves Age			
	Mean Age [a]	**Viral Load [b]**	**First Week**	**Second Week**	**Third Week**	**Fourth Week**
Diarrheic	11.9 [1]	7.99 [2]	69.0 [3]	72.1	68.8	85.7
Non-diarrheic	13.5 [1]	7.35 [2]	52.2 [3]	67.7	67.9	44.4
Total	12.7	7.67	58.8 [4]	70.6 [4,5]	68.2	52.9 [5]

[a] Mean age in days of RVA-positive calves. [b] Mean RVA viral load expressed as log10 of RVA genome copies per milliliter of feces. Equal numbers in superscript refer to values with statistically significant differences ($p < 0.05$).

2.2. VP7 and VP4 Genotyping

We obtained 58 and 116 sequences for VP7 and VP4, respectively. The detected G-types (VP7) were G6 (77.6%, 45/58), G10 (20.7%, 12/58), and G24 (1.7%, 1/58), while the P-types (VP4) were P[5] (28.4%, 33/116), P[11] (70.7%, 82/116), and P[33] (0.9%, 1/116). The following G- and P-type combinations were obtained for 57 strains: G6P[11] (40.4%, 23/57), G6P[5] (38.6%, 22/57), G10P[11] (19.3%, 11/57), and G24P[33] (1.8%, 1/57). Furthermore, 60 strains had undetermined G- or P-type: GXP[11] (80.0%, 48/60), GXP[5] (18.3%, 11/60), and G10P[X] (1.7%, 1/60).

2.3. VP6 and NSP1-5 Genotyping

Ten samples, including representative VP7 and VP4 genotype combinations observed, were selected for VP6 and NSP1-5 gene characterization: 2 G6P[5], 2 G6P[11], 2 G10P[11], 2 GXP[11], 1 G10P[X], and 1 G24P[33] (Table 2). All the strains were I2 (VP6), N2 (NSP2), and E12 (NSP4). Nine were H3 and one could not be determined HX (NSP5). Five strains were A3, four were A13, and one could not be determined AX (NSP1). Eight strains were T6, one was T9, and one could not be determined TX (NSP3).

Table 2. Genotype constellation of 10 RVA strains from Uruguayan calves.

Strain	VP7	VP4	VP6	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Cow-wt/URY/LVMS781/2015/G6P[5]	G6	P[5]	I2	AX	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS1788/2016/GxP[11]	GX	P[11]	I2	A3	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS1812/2016/G6P[5]	G6	P[5]	I2	A3	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS1837/2016/G10P[11]	G10	P[11]	I2	A13	N2	TX	E12	H3
RVA/Cow-wt/URY/LVMS2625/2016/G10P[11]	G10	P[11]	I2	A13	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS3024/2016/G24P[33]	G24	P[33]	I2	A13	N2	T9	E12	H3
RVA/Cow-wt/URY/LVMS3027/2016/G6P[11]	G6	P[11]	I2	A3	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS3031/2016/G6P[11]	G6	P[11]	I2	A3	N2	T6	E12	H3
RVA/Cow-wt/URY/LVMS3053/2016/G10P[x]	G10	P[X]	I2	A13	N2	T6	E12	HX
RVA/Cow-wt/URY/LVMS3206/2016/GxP[11]	GX	P[11]	I2	A3	N2	T6	E12	H3

Uncommon genotypes are shadowed in grey.

2.4. Phylogenetic Analyses

The phylogenetic analyses showed an intricate genetic scenario. The analyses of the VP7 gene showed that G6 and G10 Uruguayan strains clustered in two and one different lineages, respectively, with sequences obtained from cattle. Specifically, the G6P[5] Uruguayan strains clustered in one lineage (split into two sublineages) with Argentinian strains, and the G6P[11] Uruguayan strains clustered separately in a lineage with Slovenian strains (Figure 2). The G10 Uruguayan strains clustered in a lineage (split into two sublineages) with Argentinian strains (Figure 3). Brazilian G6 and G10 strains clustered separately with Uruguayan and Argentinian G6 and G10 strains.

The phylogenetic analyses of the VP4 gene showed that P[5] Uruguayan strains clustered in a lineage with Argentinian G6P[5] strains obtained from cattle, and Brazilian P[5] strains clustered separate (Figure 4). The P[11] Uruguayan strains clustered in three lineages with sequences obtained from cattle, two of the lineages were comprised of G6 and G10 Argentinian strains (and one of these lineages is split into two sublineages), and the other lineage comprised of G6P[11] Brazilian strains, although P[11] Uruguayan strains were distinct to the majority of the Brazilian P[11] strains (Figure 5).

In the phylogenetic tree of the NSP1 gene, we observed that Uruguayan strains clustered in three different genetic lineages of the genotype A3: one jointly with human strains from Paraguay and Brazil, another with Italian and Belgian human strains, and another with a goat strain from Argentina and, in one genetic lineage of the genotype A13, with an Argentinian strain from a cow (Figure S1).

The phylogenetic analysis of the NSP2 gene showed that the Uruguayan strains were clustered in two separate lineages: one with Argentinian strains from cow and goat, and the other with strains from guanaco and vicuña from Argentina and strains from humans from Australia (Figure S2).

On the other hand, the phylogenetic analysis of the NSP3 gene showed that the T6 Uruguayan strains were clustered in three sublineages within one lineage: one together with strains distributed worldwide (including vaccine strains), one with Argentinian (vicuña and guanaco), Japanese (cow), Slovenian (human), and Paraguayan (human) strains, and the third with a goat strain from Argentina and a human strain from Belgium. The T9 strain clustered with the other four T9 strains detected so far (from Japan and the USA; Figure S3).

For the NSP4 gene, we observed that besides the Uruguayan strains obtained in our study, only sequences from South America were available. The phylogenetic analysis showed that Uruguayan strains clustered in four different lineages together with strains from several host species (cows, guanacos, horses, goats, and humans), all from this subcontinent (Figure S4).

The phylogenetic analysis of the NSP5 gene showed that the Uruguayan strains were clustered in three sublineages within one lineage: one together with strains distributed worldwide in several host species), other with an Argentinian strain from a cow and a Paraguayan strain obtained from a human, and another with a strain from a guanaco from Argentina, a strain from a yak from China, and a strain from a human from Hungary (Figure S5).

Lastly, the phylogenetic analysis of the VP6 gene showed that the Uruguayan strains were clustered in three lineages: one conformed only with Uruguayan strains, another lineage with an Argentinian strain from a cow, and another lineage with South American strains from various hosts

(human, llama, sheep, and goat), Japanese strains from human and cow, and a roe deer Slovenian strain (Figure S6).

Figure 2. Maximum likelihood tree of the G6 genotype of the VP7 gene. The best nucleotide substitution model (TIM2 + I + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in red. Shimodaira–Hasegawa-approximate likelihood-ratio test (SH-aLRT) values ≥ 80 are shown.

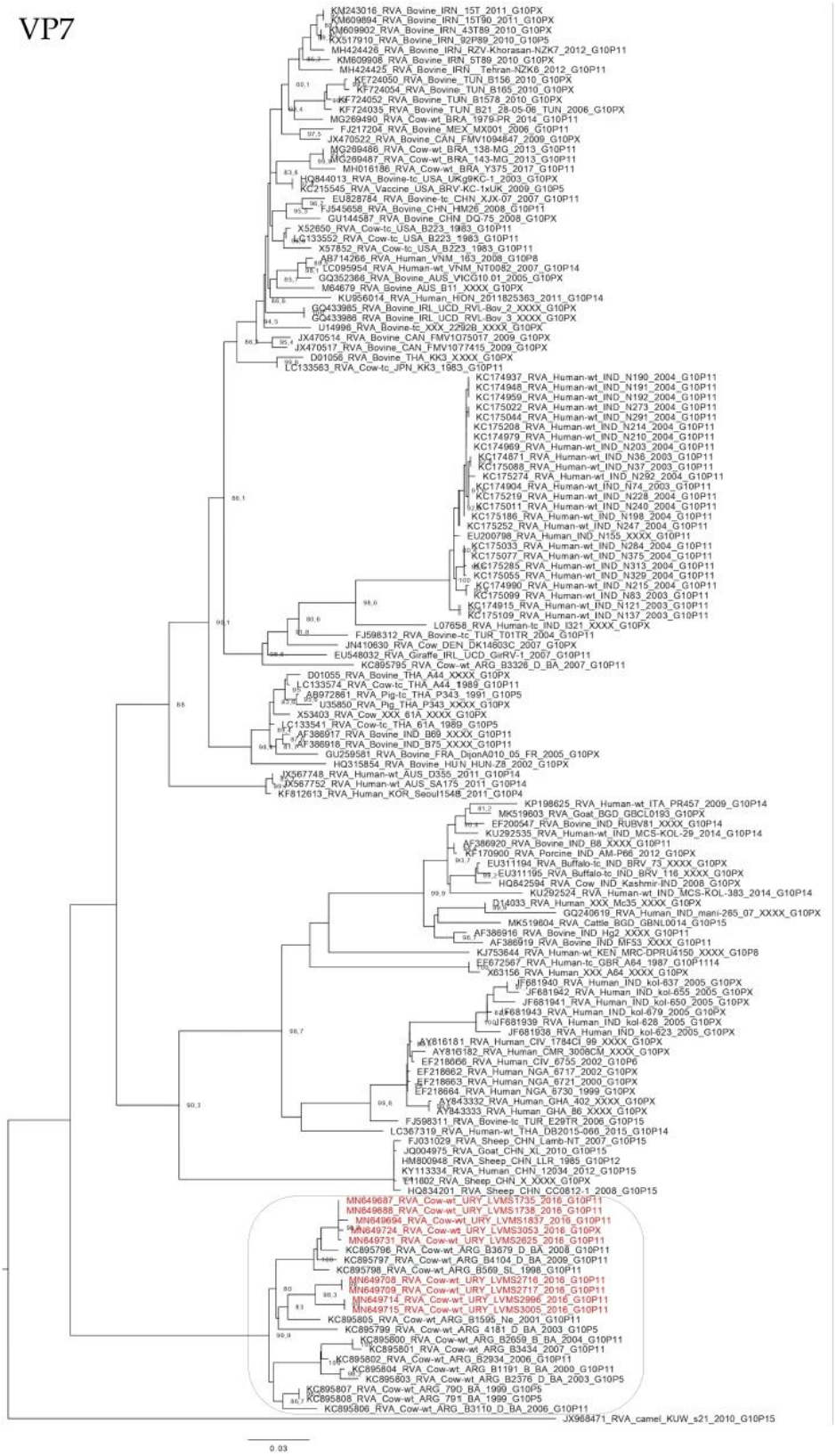

Figure 3. Maximum likelihood tree of the G10 genotype of the VP7 gene. The best nucleotide substitution model (TPM3 + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in red. SH-aLRT values ≥ 80 are shown.

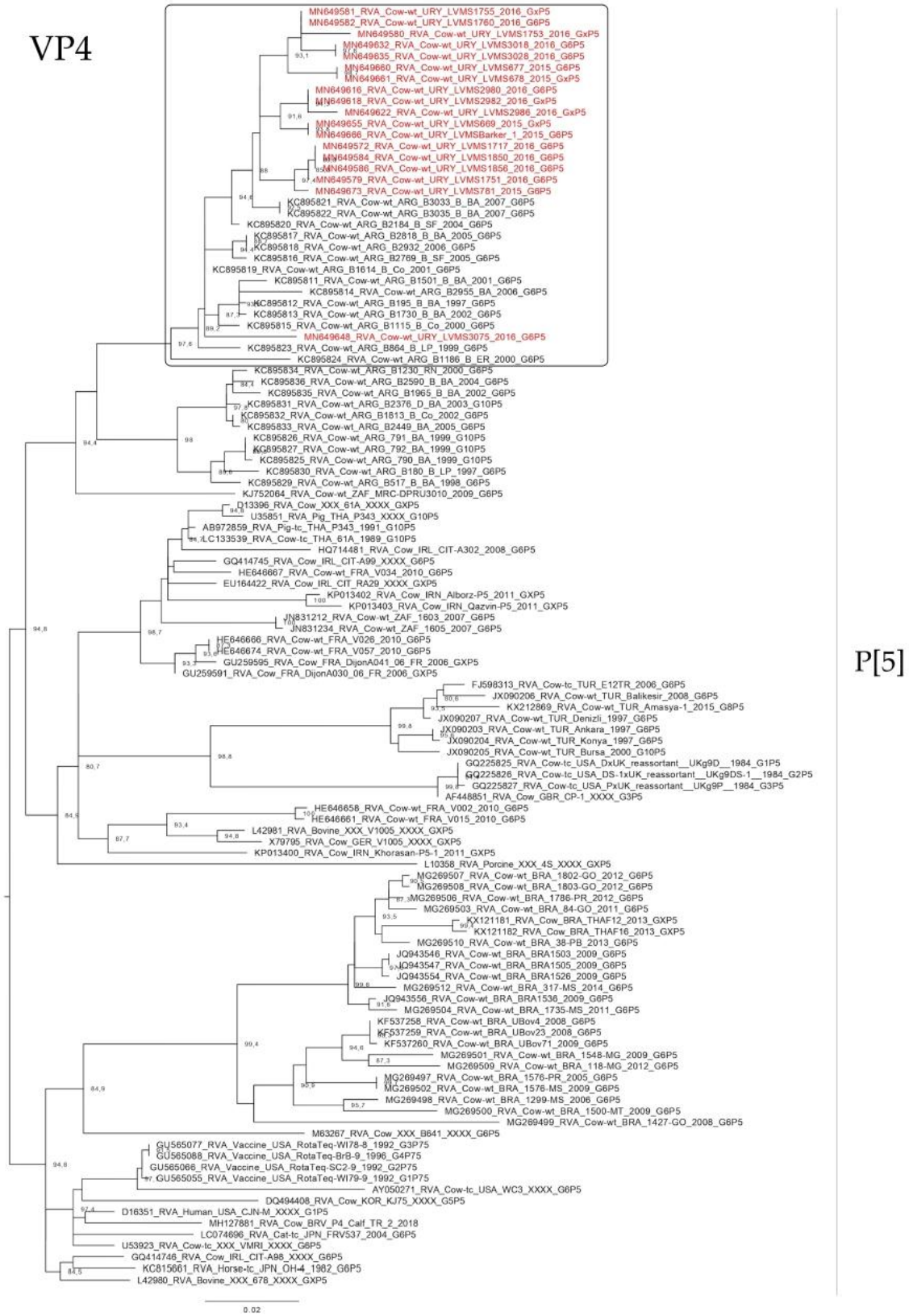

Figure 4. Maximum likelihood tree of the P[5] genotype of the VP4 gene. The best nucleotide substitution model (TIM + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in red. SH-aLRT values ≥ 80 are shown.

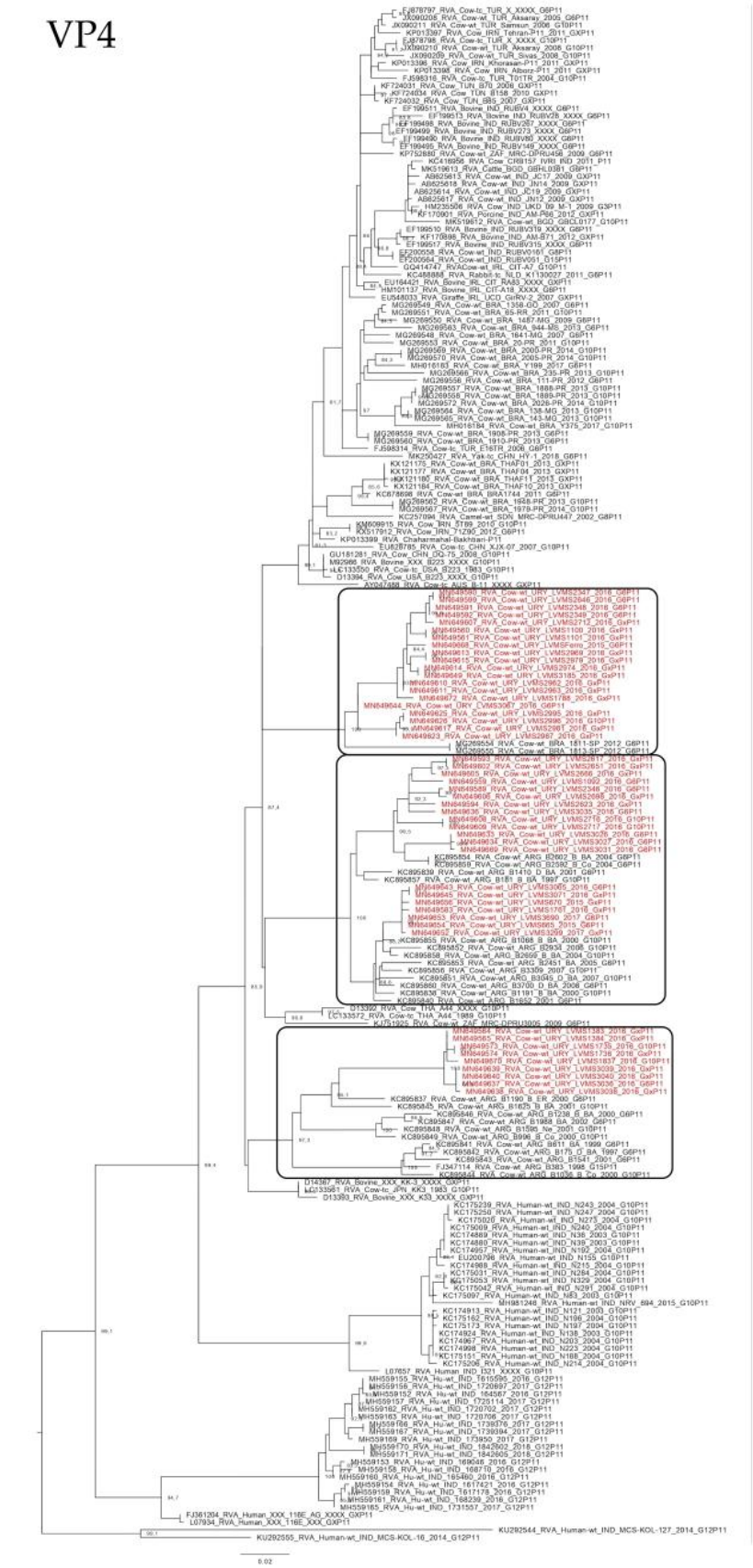

Figure 5. Maximum likelihood tree of the P[11] genotype of the VP4 gene. The best nucleotide substitution model (TPM3u + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in red. SH-aLRT values ≥ 80 are shown.

3. Discussion

Rotavirus A was detected in feces and intestinal contents collected from dairy and beef calves with a frequency of 57%, which was higher than reports from Argentina and Brazil (17–42%) [19–22], and other geographic regions (20–49%) [7,23–25]. On the other hand, in Australia, the frequency of RVA detection was 80%, which is higher than the detected in our study [6]. Interestingly, most of the mentioned studies were conducted by assays different than RT-qPCR, except the one conducted in Australia. It is well documented that the RT-qPCR for RVA detection has a higher sensitivity than other assays, reducing the risk of false-negatives (i.e., ELISA, electron microscopy, PAGE, immunochromatography, and conventional PCR) [6,26–28], which could explain the higher frequency observed in Uruguay when compared with neighboring countries while reducing the risk of false-positive results, also given its higher specificity. Furthermore, the use of RT-qPCR, which is known to detect very few genomic copies, allows pathogen detection in clinical and subclinical calves. In addition, in many field situations, the time of onset of diarrhea is not known, so the peak of pathogen shedding may have already passed, or the infection could be just settling down by the time of sampling [29]. The limit of detection in our study (10^4 gc/mL of feces) and the higher RVA viral load in diarrheic than nondiarrheic calves are in agreement with the stated by Torres-Medina et al. [29]. On the other hand, we also observed high viral loads in some nondiarrheic calves.

Infection with RVA has long been associated with diarrhea [29–31], as observed in our study, where RVA detection was more frequent in diarrheic than in nondiarrheic calves, independently of their age (up to 4 weeks). Concerning the calves' age, we observed that the proportion of calves shedding RVA was higher in the second and third weeks of age, as observed in Brazil [19,32] and elsewhere [33]. In addition, the mean age of RVA-positive calves in our study is similar to the age reported previously [31], and we observed that diarrheic calves positive for RVA were younger than nondiarrheic calves, indicating that calves are exposed to this pathogen early after birth.

Although the sampling between beef and dairy farms was unequal, our results indicate that the circulation of RVA was higher in dairy than beef calves. This contrasts with the reported results in neighboring countries, where RVA was more frequently detected in beef than dairy calves [19,20] or in a similar frequency [21]. Our results also contrast with those observed in a study conducted in Australia [6].

A common practice used to prevent NCD is the vaccination of pregnant cows/heifers during the last stage of pregnancy to protect the calves by the transference of passive maternal antibodies through colostrum intake. Most available vaccines in the Uruguayan market include bovine rotavirus A strains (most of them include two strains, G6 and G10, as detailed by the manufacturers). In this study, we observed a similar frequency of RVA detection in calves from vaccinated and unvaccinated herds. Failure in the protection against RVA infection by the vaccine was reported in studies conducted in Argentina and Brazil [34–37]; although vaccines are not effective in preventing RVA infection, they significantly reduce morbidity, the severity of diarrhea, and mortality related to RVA [38].

In this study, we determined the RVA genotypes circulating in calves in Uruguay. Overall, the VP7 and VP4 genotypes observed in this country are the most prevalent in cattle worldwide [39], although, unexpectedly, we detected a G24P[33] strain, which thus far had only been reported from an asymptomatic cow and her calf in Japan [11]. The G24P[33] strain detected in Uruguay was obtained from a 10-day-old asymptomatic dairy calf sampled in August 2016.

Regarding the VP6 and NSP1-5 genotyping, the Uruguayan strains, including the G24P[33], showed a relatively conserved genotype constellation I2-A3/A13-N2-T6/T9-E12-H3, corresponding to VP6 and NSP1-5 genotypes, respectively. These genotypes are commonly found in cattle, with the exception of T9 [40]. The T9 genotype has been sporadically detected in two cows from Japan [11], in a child from Japan [41], and in a child from the USA [42]. This genotype has been associated with atypical VP7 and VP4 genotypes (G21P[29], G24P[33], G8P[14], and G24P[14]). In this study, we observed the T9 genotype associated with G24P[33]. Indepth analysis of the RVA/Cow-wt/URY/LVMS3024/2016/G24P[33] strain revealed almost the same genotype constellation as the RVA/Cow-wt/JPN/Dai-10/2007/G24P[33] strain

from Japan, with the unusual G24, P[33], and T9 genotypes. The only difference was observed in the NSP4 gene that was E12 in the Uruguayan strain and E2 in the Japanese. It is interesting to note that all the Uruguayan strains were E12, a genotype widely detected in cattle [12], guanacos [12], horses [43,44], goats [45], and children [46,47] in South America. This reinforces the notion that the E12 genotype may be restricted to South America, as previously postulated [44].

The rare G24P[33] strain detected in our study represented a challenge. The G24, P[33], and T9 genotypes observed in this strain provides information for a possible introduction of the virus from Japan to Uruguay, or vice versa. The expansion of the Wagyu beef industry beyond Japan [48] could have influenced the dispersion of some RVA strains through live cattle exports. On the other hand, the E12 genotype in the Uruguayan G24P[33] strain and E2 genotype in the Japanese G24P[33] strain represented a probable gene reassortment, which is a more plausible scenario than the emergence of two independent strains with the same rare genotype constellation except for NSP4. Further studies should be conducted to determine the evolution and possible emergence of these rare genotypes.

In the phylogenetic analyses of all the genes, it can be observed that Uruguayan strains clustered mainly with South American strains. The only gene that did not show any South American-specific lineage was NSP3, in which the Uruguayan strains clustered mainly with Argentinian strains, but also with strains from other continents. These data, together with the identification of the E12 genotype in all the Uruguayan sequences, suggest a South American origin of RVA lineages [44]. Furthermore, the phylogenetic analyses showed an intricate pattern of diversity, with evidence of gene reassortments, interspecies transmission, local dispersion of some strains, and circulation of strains that are most prevalent in cattle worldwide.

The analyses of VP7 and VP4 showed a conserved pattern with all the Uruguayan strains clustering, with strains detected only in cattle and mainly from Argentina, indicating a probable host species and geographic linkage. Due to the shortage of G24 and P[33] sequences in the database (2 and 1, respectively), no phylogenetic analyses were performed for these genotypes. In the VP7 and VP4 phylogenetic analysis, the majority of strains characterized in this study clustered closely with strains detected in Argentinian cattle. The exceptions were one G6 lineage that clustered with European strains isolated from cattle, and one in P[11] sublineage that clustered with Brazilian strains isolated from cattle. There is a clear phylogenetic relationship between the strains detected in the cattle in Uruguay and Argentina, whereas Brazilian strains were, in general, phylogenetically distant from the Uruguayan strains. In addition, Uruguayan strains clustered together among themselves, suggesting that limited introductions of RVA into the country have occurred, but the strains were widely dispersed in the cattle. A possible explanation for the genetic similarity between the Uruguayan and Argentinian strains and their divergence to the Brazilian strains could be explained, in part, by the breed of cattle. In Uruguay and Argentina, most of the cattle breeds are *Bos taurus*, while in Brazil, there are mostly *Bos indicus* or *Bos indicus* x *Bos taurus* crosses. Although it has not been studied in cattle, different human subpopulations appeared to have different susceptibility infection and clinical disease, and this susceptibility is dependent on the rotavirus genotype, and in some cases, it also depends on different rotavirus strains of the same genotype [49].

Based on the phylogenetic analyses, we observed evidence of gene reassortment and interspecies transmission events. Regarding the former event, in addition to the previously mentioned gene reassortment of the G24P[33] strain, strong evidence was observed in the strains RVA/Cow-wt/URY/LVMS1812/2016/G6P[5] and RVA/Cow-wt/URY/LVMS3206/2016/GxP[11] because both strains clustered together in all the genes, except in VP4 (which showed different genotypes, (P[5] and P[11], respectively), indicating that a possible gene reassortment event may have occurred. Another piece of evidence was observed in the RVA/Cow-wt/URY/LVMS1788/2016/GxP[11] strain because it clustered together with other Uruguayan strains in most of the genes, except in NSP1 and NSP3 genes, which clustered alone in different genetic lineages, also suggesting a gene reassortment event. Furthermore, the strain RVA/Cow-wt/URY/LVMS1837/2016/G10P[11] clustered together with RVA/Cow-wt/URY/LVMS2625/2016/G10P[11] and RVA/Cow-wt/URY/LVMS3053/2016/G10P[x] in most of the genes, but clustered

separately in distant genetic lineages in NSP2 and NSP5; this was probably due to gene reassortment. On the other hand, an interesting observation was that, in general, G6 strains tended to cluster together in most of the genes, and the same was observed for the G10 strains, with the exceptions aforementioned.

Regarding interspecies transmission, we observed that in the analyses of VP7 and VP4, all the Uruguayan strains clustered with other bovine strains, so these gene segments seem to be more host-specific than the other genes. On the other hand, and based on the phylogenetic analyses, we observed evidence suggesting interspecies transmission because the bovine strains detected in Uruguay closely clustered with strains detected in other host species. We observed that bovine Uruguayan strains A13 (NSP1 gene) clustered together with strains isolated from humans and a goat, possibly indicating events of interspecies transmission. Two lineages showed a close relationship between Uruguayan bovine strains and human strains (from South America and Europe); these human strains were reported to be Artiodactyl-like and a product of interspecies transmission [10,47,50], as well as the goat strain of a third lineage [45], which is in accordance with our results. In the NSP2-5 and VP6 genes, we observed that the Uruguayan bovine strains clustered in some lineages with strains isolated from other host species (human, goat, guanaco, vicuna, roe deer, llama, and sheep), mainly from South America, that were proposed to be originated by interspecies transmission [12,45,47,51], again in accordance with our results. Another piece of evidence supporting this event was observed in the NSP4; all the RVA strains detected in South America were E12, independent of the host species where they were isolated (horse, cow, guanaco, human, goat), suggesting interspecies transmission and fixation of this genotype in South America [44]. The interspecies transmission of RVA is widely documented [10–13], and our results support this event. In South America, it is common to raise different livestock species on the same farm in close contact with humans [45], which increases the possibility of interspecies transmission. Our results support that interspecies transmission is a common event in South America, including the possibility of zoonotic transmission [45,51,52].

Lastly, our study had some limitations. In Uruguay, dairy farming is concentrated in the southwest region and calves are raised under intensive production systems that facilitated the collection of the samples, while beef calves are mostly bred in extensive production systems and dispersed throughout the country, which hindered the access to samples. This resulted in an overrepresentation of dairy (92%) versus beef (8%) samples in our study. Another limitation was that we had no spiked control to determine if there was inhibition of the qPCR, which may lead to false-negatives. Regarding coinfections, the methodology used has the limitation that sequences obtained from a single animal would have only represented the predominant strain and/or sequences with multiple traces that were not included in the study. It is important to mention that, from our analyses, we could not determine the route nor the time in which the gene reassortment and the interspecies transmission events took place.

4. Materials and Methods

4.1. Samples

Fecal samples of 766 live calves and intestinal contents from 67 naturally-deceased calves were collected from 833 different calves from dairy and beef herds in Uruguay between 2015 and 2018. Sampled herds were distributed in 10 of the 19 regions of the country (Figure 6), and throughout the year, including samples collected in the four climate seasons. In addition, 766 samples were from dairy calves, and 67 from beef calves. We compared the frequency of the RVA infection between groups only for dairy calves. A total of 240 dairy calves had diarrhea at the time of sampling, while 272 were nondiarrheic dairy calves (this information was unavailable for 321 calves). The distribution by age in the first, second, third, and fourth weeks of life was 148, 201, 110, and 34 dairy calves, respectively (the age was unavailable for 340 calves). A total of 225 calves were from dairy herds vaccinated against NCD and 246 calves were from nonvaccinated dairy herds (herd vaccination history was unavailable for 362 calves).

Figure 6. Map of Uruguay, the regions from which samples were collected shown in grey.

4.2. Sample Suspension, RNA Extraction, Reverse Transcription, Detection and Quantification of RVA

Samples were diluted 1:10 (*v*:*v*) in phosphate-buffered saline solution, centrifuged at 3000× *g* for 20 min at 4 °C, and supernatants were collected and stored at −80 °C. Viral RNA was extracted using a QIAamp® cador® Pathogen Mini Kit (Qiagen®, Hilden, Germany), following the manufacturer's instructions. Reverse transcription (RT) was carried out with RevertAid® Reverse Transcriptase (Thermo Fisher Scientific®, Waltham, MA, USA) and random hexamers primers (Qiagen®), following the manufacturer´s instructions. All RNAs and cDNAs were stored at −80 °C until further viral analyses. Screening and quantification of the samples for RVA identification were carried out through a quantitative polymerase chain reaction (qPCR) targeted to the NSP3 gene, as described elsewhere [9]. Briefly, 12.5 µL of SensiFAST™ Probe No-ROX Kit (Bioline®, London, UK), 5.0 µL of nuclease-free water, 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, 0.5 µL of 10 µM probe, and 5 µL of cDNA were mixed in 0.2-mL PCR tubes. All samples were analyzed in duplicate. In order to validate the complete process, an RVA-positive (G6P[5] strain) and an RVA-negative fecal sample were used as positive and negative controls, respectively.

4.3. Rotavirus A Genotyping

Quantitative-PCR positive samples were subsequently subjected to amplification of VP7 and VP4 (VP8*). Briefly, 12.5 µL of MangoMix™ (Bioline®), 5 µL of cDNA, 5.5 µL of nuclease-free water, 1 µL of dimethyl sulfoxide, 0.5 µL of 20 µM forward primer and 0.5 µL of 20 µM reverse primer were mixed in 0.2-mL PCR tubes. Forward and reverse primers for VP7 and VP4 (VP8*) amplification are described elsewhere [20,53]. In addition, 10 samples, including representative VP7 and VP4 genotype combinations observed in this study, were selected for VP6 and NSP1-5 gene characterization. Primers and cycling conditions were used, as described elsewhere [10], and PCR reagents were used, as described above. Genotyping was performed using the web-based genotyping tool RotaC v2.0 [54].

4.4. PCR Product Purification, Sequencing, and GenBank Accession Numbers

PCR products were visualized in 1–2% agarose gels and positive samples were purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®, Carlsbad, CA, USA),

according to the manufacturer's instructions. Both cDNA strands were sequenced by Macrogen Inc. (Seoul, Korea). Sequences were deposited in GenBank with accession numbers: MN649559—MN649674 (VP4), MN649675—MN649732 (VP7), MN649733—MN649742 (VP6), MN649743—MN649751 (NSP1), MN649752—MN649761 (NSP2), MN649762—MN649770 (NSP3), MN649771—MN649780 (NSP4), and MN649781—MN649789 (NSP5).

4.5. Phylogenetic Analysis

All the available sequences corresponding to the genotypes observed in the RVA strains detected in this study, previously determined with RotaC, were downloaded from the Virus Variation Resource (http://www.ncbi.nlm.nih.gov/genome/viruses/variation/) [55]. A dataset was created for each genotype, and multiple sequence alignments were obtained using Clustal W implemented in MEGA 7 software [56]. The final alignment of each gene comprised all the worldwide sequences that covered the length of the sequences obtained in this study. The length of the sequences and the nucleotide position, involved in the phylogenetic analysis of each gene, are detailed in Table 3. The nucleotide substitution models that best fit each dataset (Table 3) and the maximum likelihood trees were obtained using W-IQ-TREE (available at http://iqtree.cibiv.univie.ac.at) [57]. The branches support was estimated with the Shimodaira–Hasegawa-approximate likelihood-ratio test (SH-aLRT) [58]. Trees were visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Table 3. Information about the final alignments obtained for the phylogenetic analyses.

	NSP1	NSP2	NSP3	NSP4	NSP5	VP4 (P[5])	VP4 (P[11])	VP6	VP7 (G6)	VP7 (G10)
Sequences lenght *	1005	954	917	528	597	645	654	1143	852	837
Genomic position *	165–1169	Complete ORF	47–963	Complete ORF	Complete ORF	130–774	124–795	Complete ORF	121–972	73–909
Best nucleotide substitution model	TIM + I + G	TIM + G	TIM3 + G	HKY + G	TN + I + G	TIM + G	TPM3u + G	TIM + I + G	TIM2 + I + G	TPM3 + G

* Reference strain: WC3.

4.6. Statistical Analyses

Data were organized and graphics were generated using Microsoft® Office Excel. Categorical data were evaluated with RStudio v1.0.136 software through Pearson's chi-squared tests. Odds ratios (OR) and 95% confidence intervals (CI) were calculated with jamovi software (available at https://www.jamovi.org/). Viral load values (genome copies/milliliter of feces) were log10 transformed. For the viral load and mean age analyses, the Shapiro–Wilk test was performed, rejecting the normality of the data, so the Mann–Whitney U test was performed with the same software. For all tests, differences were considered statistically significant if the obtained p-value was < 0.05.

5. Conclusions

Rotavirus A is widespread in cattle in Uruguay and is associated with diarrhea in calves, with a peak of viral shedding at 2–3 weeks of age, and higher viral shedding in diarrheic versus non-diarrheic calves. Even though the main genotypes observed in this country are the most prevalent worldwide, a rare strain was detected with a G24-P[33]-I2-A13-N2-T9-E12-H3 genotype constellation. The E12 genotype detected in all strains, regardless of the VP7 and VP4 genotypes, appears to be a South American geographic marker. An intricate genetic scenario was evidenced, with gene reassortment and interspecies transmission events, including transmission between animals and humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/7/570/s1, Figure S1: Maximum likelihood tree of the NSP1 gene. The best nucleotide substitution model (TIM + I + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown. Figure S2: Maximum likelihood tree of the NSP2 gene. The best nucleotide substitution model (TIM + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown. Figure S3: Maximum likelihood tree of the NSP3 gene. The best nucleotide substitution model (TIM3 + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown.

Figure S4: Maximum likelihood tree of the NSP4 gene. The best nucleotide substitution model (HKY + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown. Figure S5: Maximum likelihood tree of the NSP5 gene. The best nucleotide substitution model (TN + I + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown. Figure S6: Maximum likelihood tree of the VP6 gene. The best nucleotide substitution model (TIM + I + G) and the maximum likelihood tree were obtained with W-IQ-TREE. Uruguayan strains are shown in different colors. SH-aLRT values ≥ 80 are shown.

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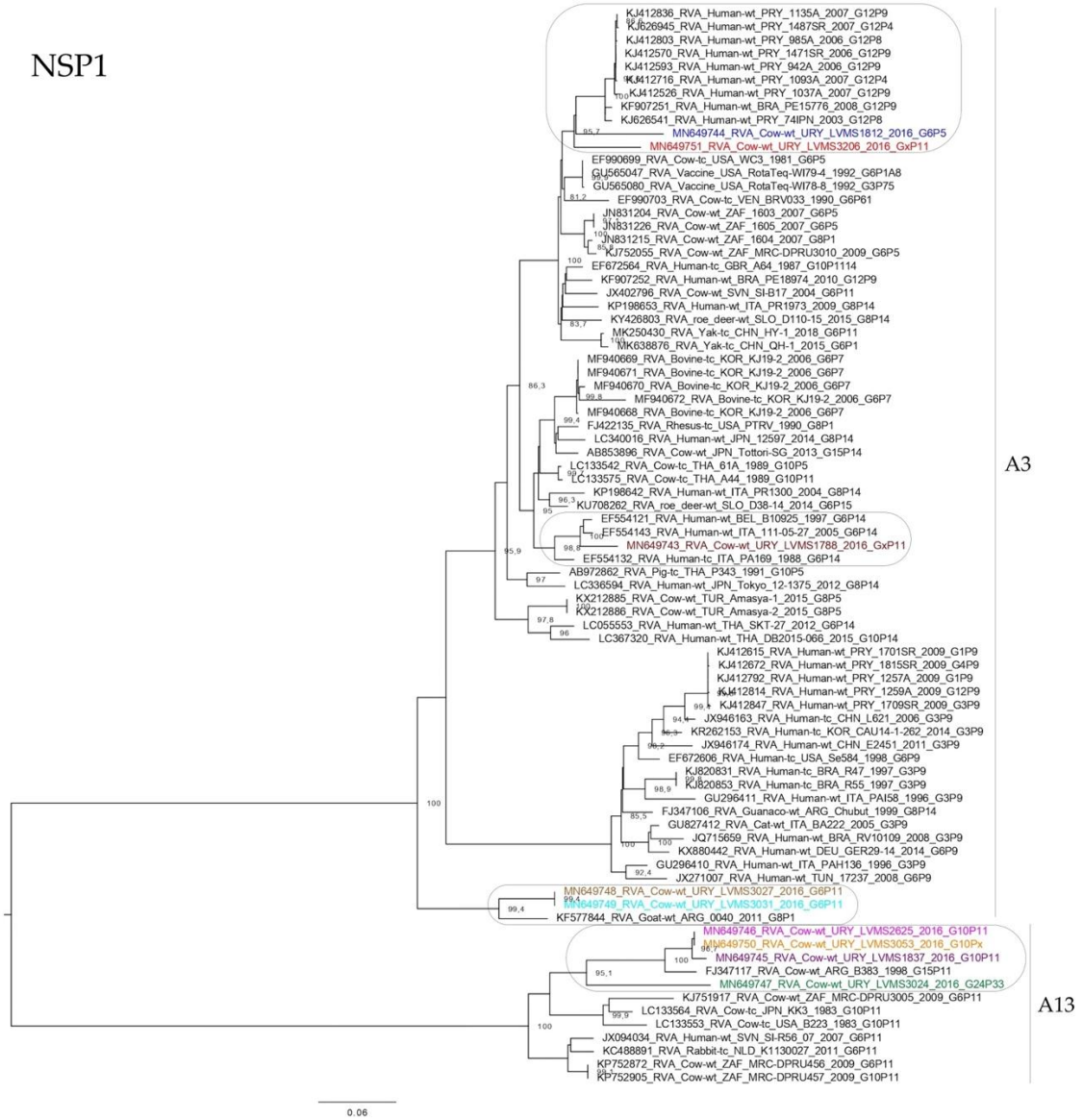
Figure S1.

NSP1



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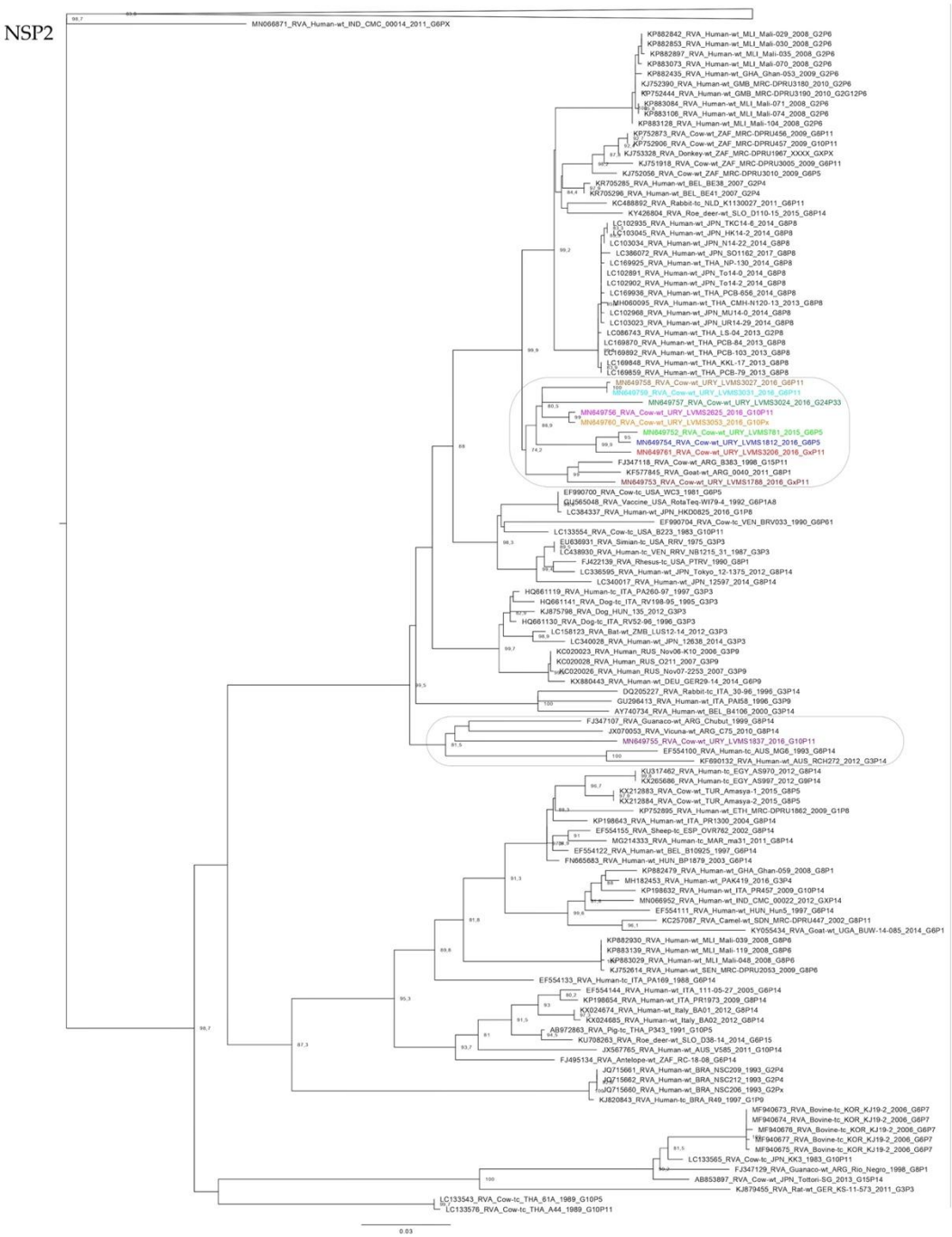
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Figure S3.

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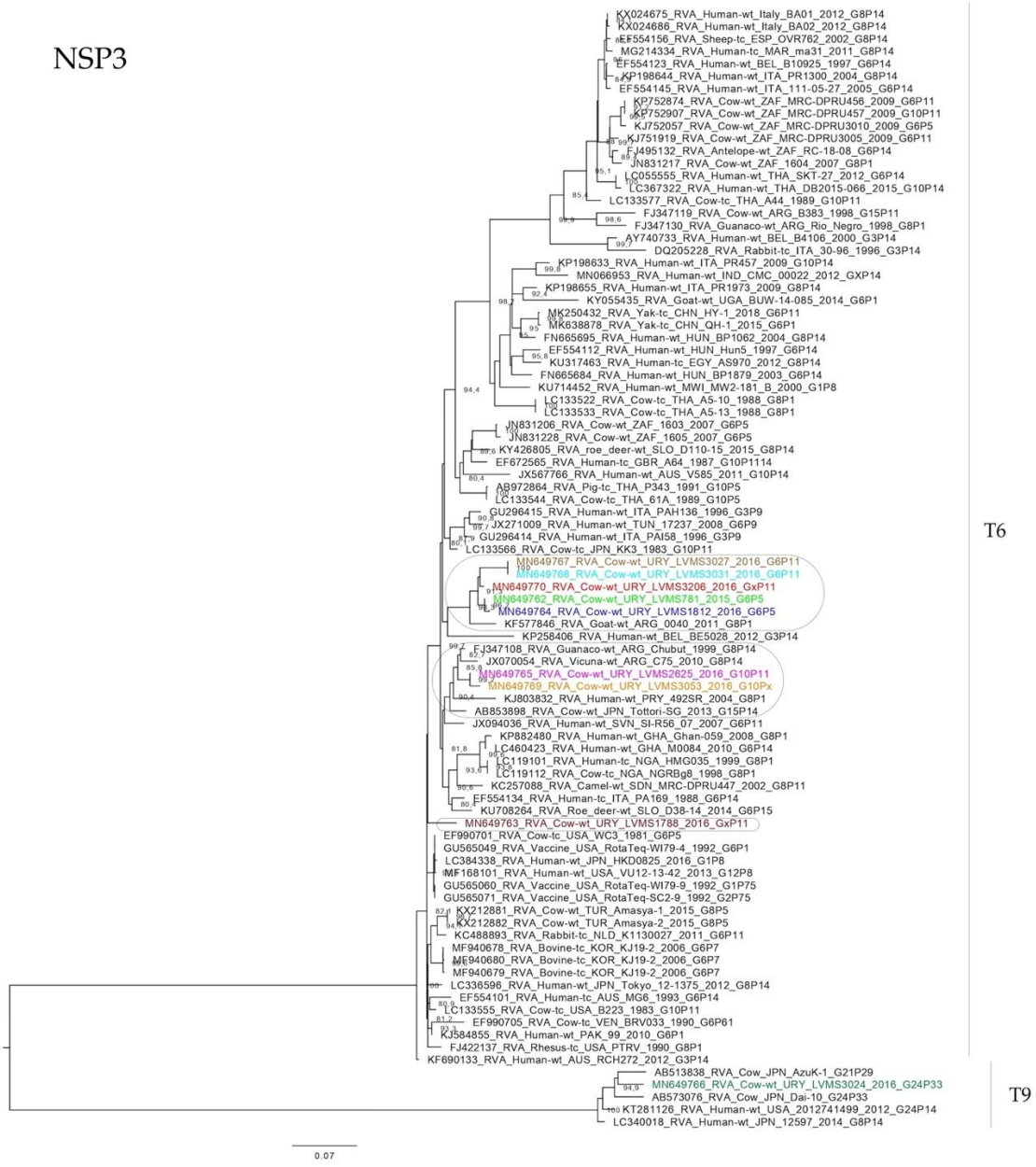

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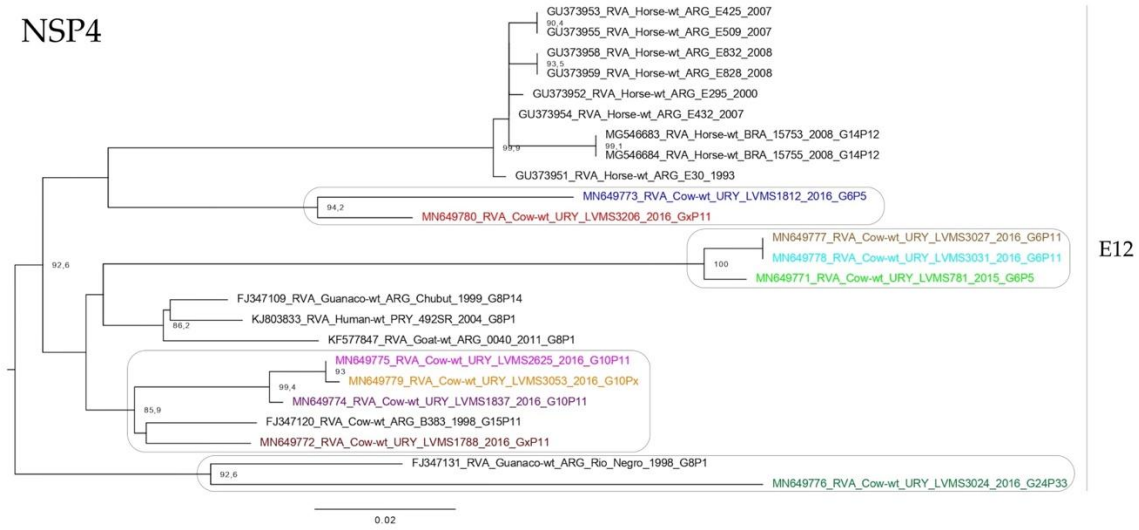
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Figure S5.

NSP5

H3

Figure S6.


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GU565059_RVA_Vaccine_USA_RotaTeq-WI79-9_1992_G1P75
GU565067_RVA_Vaccine_USA_RotaTeq-SC2-9_1992_G2P75
GU565078_RVA_Vaccine_USA_RotaTeq-WI78-8_1992_G3P75
GU565045_RVA_Vaccine_USA_RotaTeq-WI79-4_1992_G6P1A8
KT919451_RVA_Human-wt_USA_VU12-13-176_2013_G1G6P8
MF940658_RVA_Bovine-tc_KOR_KJ19-2_2006_G6P7
MF940659_RVA_Bovine-tc_KOR_KJ19-2_2006_G6P7
MF940661_RVA_Bovine-tc_KOR_KJ19-2_2006_G6P7
LC336590_RVA_Human-wt_JPN_Tokyo_12-1375_2012_G8P14
KP198649_RVA_Human-wt_ITA_PR1973_2009_G8P14
KU708258_RVA_roe_deer-wt_SLO_D38-14_2014_G6P15
EF554141_RVA_Human-wt_ITA_111-05-27_2005_G6P14
MF184821_RVA_Human-wt_IND_N259_2004_G1G10P8
MN066765_RVA_Human-wt_IND_CMC_00053_2010_G10PX
MF184850_RVA_Human-wt_IND_VR8942_2003_GXPX
JX567763_RVA_Human-wt_AUS_V585_2011_G10P14
EF583016_RVA_Human-tc_IND_69M_1980_G8P410
GU296429_RVA_Human-wt_ITA_PAI58_1996_G3P9
FJ347126_RVA_Guanaco-wt_ARG_Rio_Negro_1998_G8P1
KM276823_RVA_Alpaca-tc_PER_Alp22_2010_I2
FJ347104_RVA_Guanaco-wt_ARG_Chubut_1999_G8P14
JF805013_RVA_Human_USA_0537_2002_G3P9
EF554097_RVA_Human-tc_AUS_MG6_1993_G6P14
KF690129_RVA_Human-wt_AUS_RCH272_2012_G3P14
JX070051_RVA_Vicuna-wt_ARG_C75_2010_G8P14
LC133540_RVA_Cow-tc_THA_61A_1989_G10P5
LC133573_RVA_Cow-tc_THA_A44_1989_G10P11
KJ751926_RVA_Cow-wt_ZAF_MRC-DPRU3005_2009_G6P11
KP753064_RVA_Pig-wt_ZAF_MRC-DPRU3878_2008_G5PX
KJ752066_RVA_Cow-wt_ZAF_MRC-DPRU3010_2009_G6P5
KP752881_RVA_Cow-wt_ZAF_MRC-DPRU456_2009_G6P11
KP752914_RVA_Cow-wt_ZAF_MRC-DPRU457_2009_G10P11
LC055551_RVA_Human-wt_THA_5KT-27_2012_G6P14
KC488889_RVA_Rabbit-tc_NLD_K1130027_2011_G6P11
KX212867_RVA_Cow-wt_TUR_Amasya-1_2015_G8P5
KX212868_RVA_Cow-wt_TUR_Amasya-2_2015_G8P5
MN649733_RVA_Cow-wt_URY_LVMS781_2015_G6P5
MN649742_RVA_Cow-wt_URY_LVMS3206_2016_GxP11
MN649734_RVA_Cow-wt_URY_LVMS1788_2016_GxP11
MN649739_RVA_Cow-wt_URY_LVMS3027_2016_G6P11
MN649740_RVA_Cow-wt_URY_LVMS3031_2016_G6P11
MN649735_RVA_Cow-wt_URY_LVMS1812_2016_G6P5
AY740737_RVA_Human-wt_BEL_B4106_2000_G3P14
DQ205226_RVA_Rabbit-tc_ITA_30-96_1996_G3P14
KJ803839_RVA_Human-wt_PRY_492SR_2004_G8P1
MN649738_RVA_Cow-wt_URY_LVMS3024_2016_G24P33
AB853894_RVA_Cow-wt_JPN_Tottori-SG_2013_G15P14
KY972029_RVA_Llama-wt_PER_L27_2014_G1P8
KY972109_RVA_Sheep-wt_PER_OVI4_2014_G1P8
KY426813_RVA_roe_deer-wt_SLO_D110-15_2015_G8P14
LC340012_RVA_Human-wt_JPN_12597_2014_G8P14
KF577842_RVA_Goat-wt_ARG_0040_2011_G8P1
DQ146695_RVA_Human-tc_PHL_L26_1987_G12P4
EF583036_RVA_Human-tc_PHL_L26_1987_G12P4
DQ870488_RVA_Human-tc_JPN_S2_1980_G2P4
DQ870507_RVA_Human-tc_USA_DS-1_1976_G2P4
KC178799_RVA_Human_ITA_PAI11_1996_G2P4
MN649737_RVA_Cow-wt_URY_LVMS2625_2016_G10P11
MN649741_RVA_Cow-wt_URY_LVMS3053_2016_G10Px
MN649736_RVA_Cow-wt_URY_LVMS1837_2016_G10P11
FJ347115_RVA_Cow-wt_ARG_B383_1998_G15P11
KC815684_RVA_Horse-tc_JPN_BI_1981_G3P12
KC815697_RVA_Horse-tc_JPN_CH-3_1987_G14P12
JF712581_RVA_Horse-wt_ARG_E403_2006_G14P12
KU317470_RVA_Human-tc_EGY_AS970_2012_G8P14
KX265694_RVA_Human-tc_EGY_AS997_2012_G9P14
KJ752132_RVA_Human-wt_ETH_MRC-DPRU1844-08_2008_G3P6
EF554108_RVA_Human-wt_HUN_Hun5_1997_G6P14
KP198638_RVA_Human-wt_ITA_PR1300_2004_G8P14
GU296428_RVA_Human-wt_ITA_PAH136_1996_G3P9
LC119097_RVA_Human-tc_NGA_HMG035_1999_G8P1
LC119108_RVA_Cow-tc_NGA_NGRBg8_1998_G8P1
GU827410_RVA_Cat-wt_ITA_BA222_2005_G3P9
GQ477087_RVA_Human_RUS_Nov06-K10_2006_G3P9
JX867343_RVA_Human_RUS_O211_2007_G3P9
KF036169_RVA_Human_RUS_O1180_2011_G3P9
GQ477086_RVA_Human_RUS_Nov06-K2_2006_G3P9
JX867342_RVA_Human_Hu_RUS_O202_2007_G3P9
JQ230092_RVA_Human_RUS_Nov10-N507_G3P9
KX880438_RVA_Human-wt_DEU_GER29-14_2014_G6P9
JX867344_RVA_Human_RUS_O1154_2011_G3P9
HM113528_RVA_Human_EGY_Egy3399_2004_G6P14
JQ230102_RVA_Human_RUS_Omsk08-442_2008_G3P9
GQ477091_RVA_Human_RUS-Nov07-2253
I2
0.04

B.4. Estudio de astrovirus bovino en muestras clínicas

Artículo 5. Vigilancia de astrovirus bovino en Uruguay revela una alta tasa de detección de una nueva especie de *Mamastrovirus*

Este artículo se enfocó en la vigilancia de BoAstV en Uruguay, determinando que el virus se encuentra circulando en nuestro país con una diversidad genética importante. Además, la principal especie de BoAstV detectada, no estaba incluida en la clasificación taxonómica del virus, por lo que se hicieron los estudios necesarios para determinar que se trataba de una especie distinta a las previamente descritas, confirmando que efectivamente cumplía con los requisitos para ser incluida como una nueva especie. En concreto, 3 especies fueron detectadas, posiblemente con una cuarta especie también presente (o en su defecto una cepa recombinante). El virus fue detectado en la misma frecuencia tanto en materia fecal como contenido intestinal. Además, se observó una gran correlación en cuanto a la clasificación utilizando una región parcial de la polimerasa o utilizando la cápside completa, permitiendo utilizar un fragmento más pequeño pero igual de informativo, disminuyendo el costo, así como también la sensibilidad de los resultados ya que la polimerasa es más conservada que la cápside.

Estos resultados fueron publicados en la revista Viruses: **Castells M**, Bertoni E, Caffarena RD, Casaux ML, Schild C, Victoria M, Riet- Correa F, Giannitti F, Parreño V, Colina R. Bovine Astrovirus Surveillance in Uruguay Reveals High Detection Rate of a Novel Mamastrovirus Species. Viruses. 2019a Dec 27;12(1):32. doi: 10.3390/v12010032.

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Bovine Astrovirus Surveillance in Uruguay Reveals High Detection Rate of a Novel *Mamastrovirus* Species

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Abstract: Viral infections affecting cattle lead to economic losses to the livestock industry worldwide, but little is known about the circulation, pathogenicity and genetic diversity of enteric bovine astrovirus (BoAstV) in America. The aim of this work was to describe the prevalence and genetic diversity of enteric BoAstV in dairy cattle in Uruguay. A total of 457 fecal and 43 intestinal contents from dairy calves were collected between July 2015 and May 2017 and tested by RT-PCR, followed by sequencing and phylogenetic analyses of the polymerase and capsid regions. Twenty-six percent (128/500) of the samples were positive. Three different species within the *Mamastrovirus* genus were identified, including *Mamastrovirus 28*, *Mamastrovirus 33* (3 samples each) and an unclassified *Mamastrovirus* species (19 samples). The unclassified species was characterized as a novel *Mamastrovirus* species. BoAstV circulates in Uruguayan dairy cattle with a high genetic diversity. The eventual clinicopathological significance of enteric BoAstV infection in cattle needs further investigation.

Keywords: bovine astrovirus; dairy cattle; genetic diversity; prevalence; Uruguay; Mamastrovirus species

1. Introduction

Astroviruses (AstVs) are small, non-enveloped viruses, of 28 to 30 nm in diameter, with distinctive five- or six-pointed, star-like virions. The genome is composed of positive-sense, single-stranded RNA of 6.3–7.9 kb that includes three open reading frames (ORFs). AstVs are members of the *Astroviridae* family, which is further divided in two genera: *Mamastrovirus* (MAstV) and *Avastrovirus*, infecting mammals and birds, respectively. Bovine astroviruses (BoAstVs) are members of the MAstV genus, detected for the first time in 1978, in calves with enteritis [1]. Initially, BoAstVs did not capture much

attention in the scientific community, due to their inability to cause diarrhea in experimentally infected gnotobiotic calves [1], although it was proven that they are indeed capable of infecting the dome epithelial cells of the ileum [2]. In coinfections with other viruses, such as rotavirus and torovirus, the infectious capacity of BoAstV seems to be increased [2], although whether this is clinically relevant, or the virus plays a synergistic role in the development of diarrhea in cattle, needs further clarification. In 2013, the association of a genetically different lineage of BoAstV with neurologic disease and encephalomyelitis in cattle [3], which was recently described in Uruguay [4], resulted in increased interest in studying this group of viruses, along with their molecular epidemiology and pathogenicity. Recently, BoAstV was detected in 4/50 calves with bovine respiratory disease (BRD) and was absent in 50 asymptomatic calves in a case control study, but this viral infection was not statistically associated with BRD [5]. The role of AstV in respiratory disease remains unclear, but there is increasing evidence of detection of AstV in respiratory samples from humans and animals with acute respiratory disease [5–8].

MAstV is divided into viral species based on genetic differences higher than approximately 0.30 to 0.35 at the protein level in the complete capsid sequence [9]. The current classification of the MAstV genus according to the International Committee on Taxonomy of Viruses (ICTV), includes 19 species (namely MAstV-1 through 19) (https://talk.ictvonline.org/taxonomy/, last access 25th October 2019), although there are up to 33 tentative species, not yet confirmed by the ICTV [9,10]. BoAstVs have not yet been assigned to species. Nevertheless, it has been proposed that they belong to at least six different species: MAstV-13, MAstV-24, MAstV-28, MAstV-29, MAstV-30 and MAstV-33, that are closely related to other AstVs from various host species, indicating possible interspecies transmission events [10,11]. Two serotypes have been recognized [12], but current phylogenetic evidence indicates that the diversity of BoAstVs circulating in cattle is probably greater [10]. The aim of this study was to determine the prevalence of enteric infection by BoAstV and its genetic diversity in Uruguayan dairy cattle.

2. Materials and Methods

2.1. Samples, RNA Extraction and Reverse Transcription

A total of 500 samples (457 fecal and 43 intestinal contents) obtained from calves belonging to dairy herds of the Uruguayan dairy basin, were collected between July 2015 and May 2017. Samples were diluted 1:10 (*v:v*) in phosphate-buffered saline solution, centrifuged at 3000× *g* for 20 min at 4 °C, and supernatants were collected and stored at −80 °C. Viral RNA was extracted from each sample using QIAamp® cador® Pathogen Mini Kit (Qiagen®, Hilden, Germany), following the manufacturer's instructions. Reverse transcription (RT) was carried out with RevertAid® Reverse Transcriptase (Thermo Fischer Scientific®, Waltham, MA, USA) and random hexamers primers (Qiagen®). All RNAs and cDNAs were stored at −20 °C.

2.2. Polymerase Chain Reaction for BoAstV Detection and Sequencing

Polymerase chain reactions (PCR) were performed using MangoMix™ (Bioline®, London, UK) and primers that amplify a 432-nucleotide fragment of the polymerase gene of AstV. Briefly, 12.5 µL of MangoMix™, 5 µL of cDNA, 4.5 µL of nuclease-free water, 1 µL of dimethyl sulfoxide, 1 µL of 10 µM BoAstV-F primer and 1 µL of 10 µM BoAstV-R primer were mixed in 0.2 mL PCR tubes. PCR primers sequences and cycling conditions were the same as those described by Tse et al. [13]. PCR products were visualized in 2% agarose gels, 25 (20%). PCR-positive products were purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®, Carlsbad, CA, USA), according to the manufacturer's instructions, and both DNA strands were sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were deposited in GenBank with accession numbers MH123895-MH123814 and MH123817-MH123921.

2.3. Capsid PCR and Sequencing

Complete capsid amplification of strains LVMS681 and LVMS2704 was performed. Primers were designed in order to amplify complete capsid (Table 1), using sequences obtained from GenBank. Briefly, 12.5 µL of MangoMix™, 5 µL of cDNA, 4.5 µL of nuclease-free water, 1 µL of dimethyl sulfoxide, 1 µL of 10 µM primer forward and 1 µL of 10 µM primer reverse were mixed in 0.2 mL PCR tubes and subjected to an initial step of 5 min at 95 °C, followed by 40 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1.5 min, ending with 10 min at 72 °C for final extension. PCR products were visualized in 1–2% agarose gels, purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®), according to the manufacturer's instructions, and both DNA strands were sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were deposited in GenBank with accession numbers MN200262 and MN200263. In addition, six partial capsid sequences were obtained and deposited in GenBank with accession numbers MN231236-MN231241.

Table 1. Primers used for complete capsid amplification.

Primer Name	5′–3′ Sequence	Genomic Region	Reference
BoAstV-F BoAstV-R	GAYTGGACBCGHTWTGATGG KYTTRACCCACATNCCAA	RdRp	[13]
BoAstV-CAP-U-33F1 BoAstV-CAP-U-33R1	GCCCTCTATGGGAAACTCCT GTMACCAKCCAKATWATYTC	Capsid	This study
BoAstV-CAP-UF2 BoAstV-CAP-UR2	CAACARCCWGGGTTYATGAA ATCCTCATCAGAGAGATCA	Capsid	This study
BoAstV-CAP-UF3 BoAstV-CAP-UR3	TTGGAGATGGCRGAYGATGA GCCAAATTAAATTAACTGG	Capsid	This study
BoAstV-CAP-28F1 BoAstV-CAP-28R1	AGCCTCTGTGGGAAACTAGA CCACCAVCCRCCCHTRAAAAGCCA	Capsid	This study

2.4. Phylogenetic Analyses

Available sequences of a partial polymerase genomic region of BoAstV and related AstVs from other hosts (344 nucleotides from position 3289 to 3620, reference strain accession number: NC_023631) were downloaded from the GenBank database (Table S1).

For MAstV classification, complete capsid sequences were downloaded from GenBank database (Table S2). The datasets of nucleotide and amino acid sequences were aligned using Clustal W implemented in MEGA 7 software [14]. The substitution model that best fit each alignment and maximum likelihood trees (with the substitution model selected previously) were obtained with W-IQ-TREE (available at http://iqtree.cibiv.univie.ac.at) [15].

2.5. Estimates of the Evolutionary Divergence between Sequences

Amino acid p-distances were estimated with MEGA 7 software [14]. Pairwise distances were estimated with the default parameters of MEGA version 7.0.21, and the alignment of complete capsid amino acid sequences, obtained previously for the phylogenetic analysis, was used as input.

3. Results

BoAstV was detected in 26% (128/500) of the samples screened by PCR targeting the polymerase genomic region. The same frequency of detection was observed in feces (26%, 117/457) and intestinal contents (26%, 11/43). Phylogenetic analysis with the partial polymerase genomic region showed that the Uruguayan strains clustered in three groups: 3/25 (12%) with MAstV-28, and 3/25 (12%) with MAstV-33, while 19/25 (76%) strains formed a monophyletic group with other unclassified strains from all over the world (Figure 1).

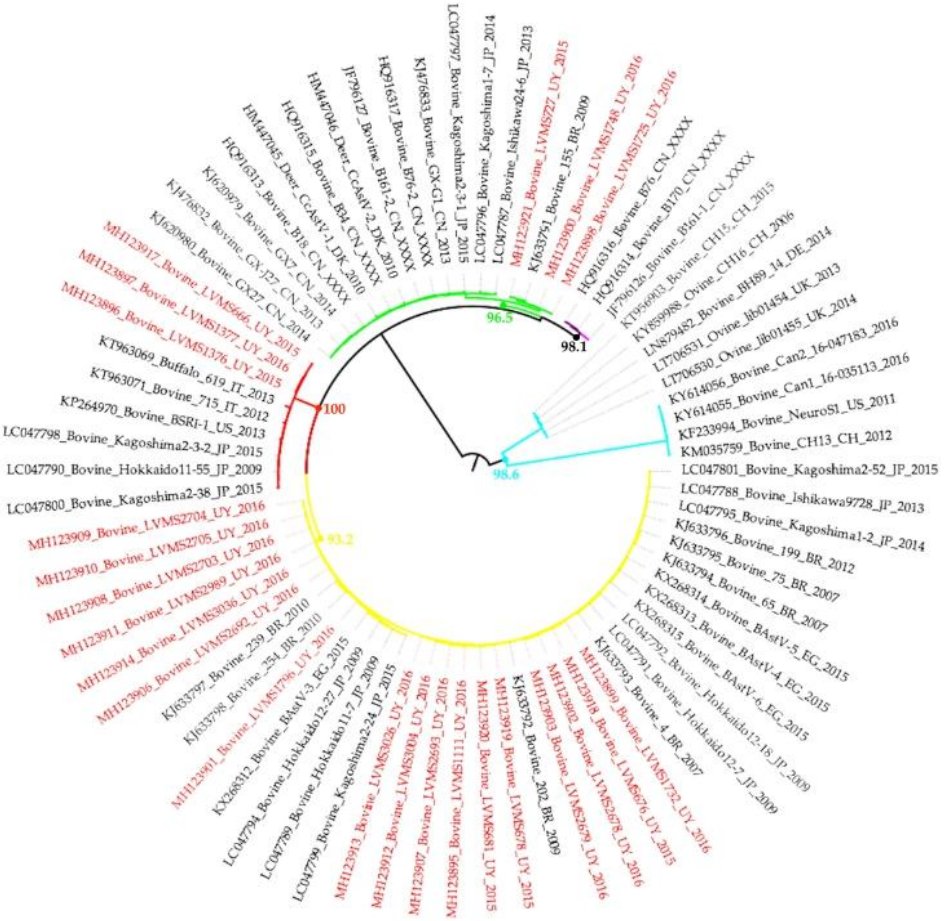

Figure 1. Maximum-likelihood tree constructed with the nucleotide sequences of the partial polymerase genomic region used for BoAstV screening. A 432-nucleotide fragment of the polymerase gene was used for the analysis. TIM2e (AC = AT, CG = GT and equal base frequencies) plus R (FreeRate model, that generalizes the plus Gamma model by relaxing the assumption of Gamma-distributed rates) was used as the nucleotide substitution model that best fitted the data. Branch colors indicate the assigned MAstV species; MAstV-13 (light blue), MAstV-28 (red), MAstV-29 (pink), MAstV-30 (violet), MAstV-33 (green) and MAstV-Unclassified (yellow). Names in red font correspond to Uruguayan strains. Bootstrap values for key nodes are indicated.

The phylogenetic analyses with the complete capsid sequences confirmed the clustering of the Uruguayan strains LVMS681 and LVMS2704 in a group with other unclassified strains, both at amino acid and nucleotide levels (Figure 2 and Figure S1). These results were confirmed by the analysis of the p-distance in the complete capsid sequence at the protein level (Table 2). Based on these analyses, LVMS681 and LVMS2704 should be assigned to a new MAstV species by the ICTV.

Based on partial capsid sequences, six additional Uruguayan strains were classified; four within the unclassified MAstV species, one within MAstV-28 and one within MAstV-29 (Figure S2). Uruguayan strains of the unclassified MAstV species showed an insertion of six amino acids (positions 54–59 of the LVMS2704 complete capsid amino acid sequence), when compared with Japanese strains of this species available in the database (Figure 3). This region is characterized by a repetitive sequence of polar uncharged amino acids (mainly glutamine) followed by a repetitive sequence of basic amino acids (mainly arginine); the insertion is in the polar uncharged region and is mainly formed of glutamines (Figure 3). In addition, a deletion of one amino acid was observed in LVMS681 and LVMS2704 (between positions 69 and 70 of the LVMS2704 complete capsid amino acid sequence) (Figure 3).

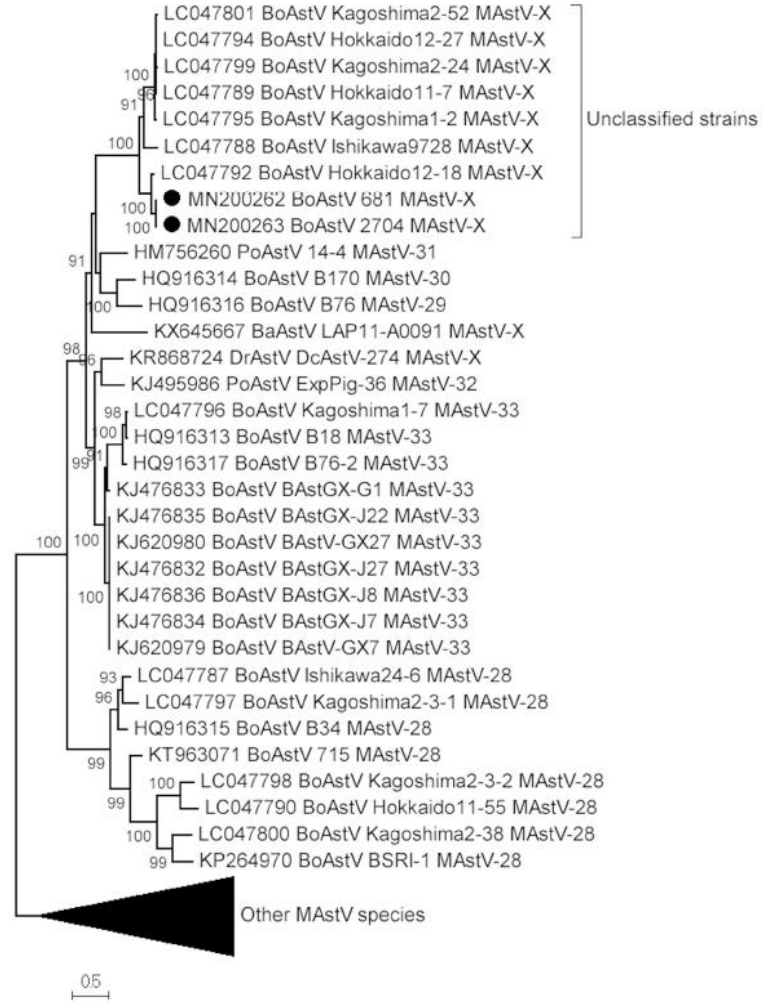

Figure 2. Maximum-likelihood tree constructed with complete capsid amino acid sequences. LG (General matrix) + gamma (with four categories of rates to approximate the gamma distribution) + invariant sites (allows a proportion of invariable sites) was used as the amino acid substitution model that best fit the data. All the assigned and tentatively assigned species of MAstV were included in the analysis (see Table S2); some were condensed for better visualization. The two Uruguayan strains are indicated with black-filled circles. The clade with the unclassified strains is shown. aLRT values higher than 90 are shown.

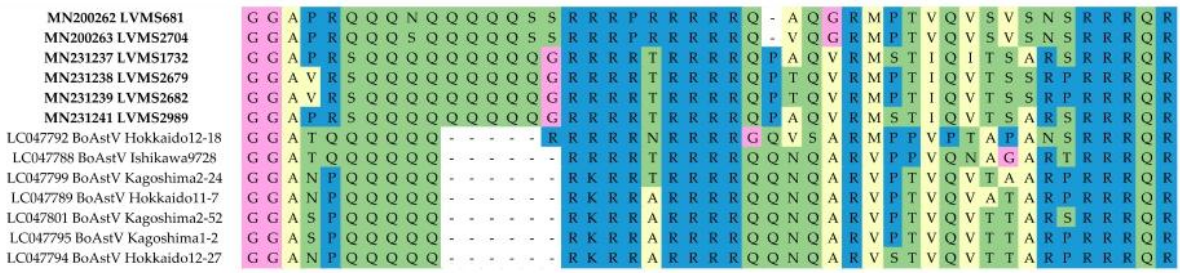

Figure 3. Amino acid alignment of unclassified strains. The amino acid alignment from position 44 to 89 of the capsid protein (LVMS2704 used as reference) is shown.

Table 2. Estimates of evolutionary divergence between sequences at the amino acid level. The number of amino acid differences per site between sequences are shown (p-distance), on the left side the reference is the Uruguayan strain LVMS2704 (MN200263), and on the right side the reference is the Uruguayan strain LVMS681 (MN200262). Complete capsid sequences were used. Sequence names are: Accession number Host Strain name MAstV-species.

Uruguayan Strain LVMS2704 (MN200263)		Uruguayan Strain LVMS681 (MN200262)	
MAstV-Species	**Distance**	**MAstV-Species**	**Distance**
MN200262 BoAstV LVMS681 MAstV-X	0.01	MN200263 BoAstV LVMS2704 MAstV-X	0.01
LC047792 BoAstV Hokkaido12-18 MAstV-X	0.09	LC047792 BoAstV Hokkaido12-18 MAstV-X	0.09
LC047794 BoAstV Hokkaido12-27 MAstV-X	0.29	LC047794 BoAstV Hokkaido12-27 MAstV-X	0.29
LC047795 BoAstV Kagoshima1-2 MAstV-X	0.29	LC047795 BoAstV Kagoshima1-2 MAstV-X	0.29
LC047801 BoAstV Kagoshima2-52 MAstV-X	0.29	LC047801 BoAstV Kagoshima2-52 MAstV-X	0.29
LC047789 BoAstV Hokkaido11-7 MAstV-X	0.29	LC047789 BoAstV Hokkaido11-7 MAstV-X	0.29
LC047799 BoAstV Kagoshima2-24 MAstV-X	0.30	LC047799 BoAstV Kagoshima2-24 MAstV-X	0.30
LC047788 BoAstV Ishikawa9728 MAstV-X	0.31	LC047788 BoAstV Ishikawa9728 MAstV-X	0.31
HQ916314 BoAstV B170 MAstV-30	0.48	HQ916314 BoAstV B170 MAstV-30	0.48
HQ916316 BoAstV B76 MAstV-29	0.49	HQ916316 BoAstV B76 MAstV-29	0.49
HM756260 PoAstV 14-4 MAstV-31	0.52	HM756260 PoAstV 14-4 MAstV-31	0.52
KJ476833 BoAstV BAstGX-G1 MAstV-33	0.54	KJ476833 BoAstV BAstGX-G1 MAstV-33	0.54
KX645667 BaAstV LAP11-A0091 MAstV-X	0.54	KX645667 BaAstV LAP11-A0091 MAstV-X	0.54
LC047796 BoAstV Kagoshima1-7 MAstV-33	0.55	LC047796 BoAstV Kagoshima1-7 MAstV-33	0.54
HQ916313 BoAstV B18 MAstV-33	0.55	HQ916313 BoAstV B18 MAstV-33	0.55
KJ495986 PoAstV ExpPig-36 MAstV-32	0.55	HQ916317 BoAstV B76-2 MAstV-33	0.55
HQ916317 BoAstV B76-2 MAstV-33	0.55	KJ495986 PoAstV ExpPig-36 MAstV-32	0.55
KJ620979 BoAstV BAstV-GX7 MAstV-33	0.55	KJ620979 BoAstV BAstV-GX7 MAstV-33	0.55
KJ476834 BoAstV BAstGX-J7 MAstV-33	0.55	KJ476834 BoAstV BAstGX-J7 MAstV-33	0.55
KJ476832 BoAstV BAstGX-J27 MAstV-33	0.55	KJ476832 BoAstV BAstGX-J27 MAstV-33	0.55
KJ476836 BoAstV BAstGX-J8 MAstV-33	0.55	KJ476836 BoAstV BAstGX-J8 MAstV-33	0.55
KJ620980 BoAstV BAstV-GX27 MAstV-33	0.55	KJ620980 BoAstV BAstV-GX27 MAstV-33	0.55
KJ476835 BoAstV BAstGX-J22 MAstV-33	0.55	KJ476835 BoAstV BAstGX-J22 MAstV-33	0.55
KR868724 DrAstV DcAstV-274 MAstV-X	0.57	KR868724 DrAstV DcAstV-274 MAstV-X	0.56
HQ916315 BoAstV B34 MAstV-28	0.60	HQ916315 BoAstV B34 MAstV-28	0.60
LC047787 BoAstV Ishikawa24-6 MAstV-28	0.61	LC047787 BoAstV Ishikawa24-6 MAstV-28	0.61
LC047797 BoAstV Kagoshima2-3-1 MAstV-28	0.61	LC047797 BoAstV Kagoshima2-3-1 MAstV-28	0.61
LC047800 BoAstV Kagoshima2-38 MAstV-28	0.65	LC047800 BoAstV Kagoshima2-38 MAstV-28	0.65
KT963071 BoAstV 715 MAstV-28	0.66	KT963071 BoAstV 715 MAstV-28	0.66
LC047798 BoAstV Kagoshima2-3-2 MAstV-28	0.67	LC047798 BoAstV Kagoshima2-3-2 MAstV-28	0.67
KP264970 BoAstV BSRI-1 MAstV-28	0.67	KP264970 BoAstV BSRI-1 MAstV-28	0.67
LC047790 BoAstV Hokkaido11-55 MAstV-28	0.68	LC047790 BoAstV Hokkaido11-55 MAstV-28	0.68
JQ408745 MuAstV TF18LM MAstV-X	0.70	JX684071 PoAstV US-P2011-1 MAstV-26	0.70
JX544746 MuAstV STL 4 MAstV-X	0.70	JQ408745 MuAstV TF18LM MAstV-X	0.70
JX684071 PoAstV US-P2011-1 MAstV-26	0.70	JX544746 MuAstV STL 4 MAstV-X	0.70
KU764486 PoAstV 15-12 MAstV-26	0.71	KU764486 PoAstV 15-12 MAstV-26	0.71
JX556692 PoAstV IL135 MAstV-27	0.72	JX556692 PoAstV IL135 MAstV-27	0.72
KM017742 CaAstV FAstV-D2 MAstV-2	0.74	KM017742 CaAstV FAstV-D2 MAstV-2	0.74
L23513 HuAstV Oxford-1 MAstV-1	0.74	JN592482 OvAstV OAstV-2 MAstV-24	0.74
JN592482 OvAstV OAstV-2 MAstV-24	0.74	LC201619 PoAstV Ishi-Im1-1 MAstV-24	0.74
LC201619 PoAstV Ishi-Im1-1 MAstV-24	0.74	L23513 HuAstV Oxford-1 MAstV-1	0.74
HM450381 RatAstV RS118 MAstV-25	0.75	HM450381 RatAstV RS118 MAstV-25	0.75
L06802 HuAstV Oxford-2 MAstV-1	0.75	LC047793 BoAstV Hokkaido12-25 MAstV-24	0.75
LC047793 BoAstV Hokkaido12-25 MAstV-24	0.76	GQ914773 PoAstV Shanghai MAstV-3	0.75
GQ914773 PoAstV Shanghai MAstV-3	0.76	L06802 HuAstV Oxford-2 MAstV-1	0.76
JN420351 CslAstV 4 1136 MAstV-11	0.76	KR349491 DoAstV Grav MAstV-5	0.76
KR349491 DoAstV Grav MAstV-5	0.76	JN420351 CslAstV 4 1136 MAstV-11	0.76
JN420354 CslAstV 1169 MAstV-4	0.76	JN420354 CslAstV 1169 MAstV-4	0.76
FJ222451 HuAstV MLB1 MAstV-6	0.77	FJ222451 HuAstV MLB1 MAstV-6	0.77
FJ571068 BaAstV LS11 MAstV-17	0.78	JF729316 RabAstV 2208 MAstV-23	0.78
JF729316 RabAstV 2208 MAstV-23	0.78	FJ571068 BaAstV LS11 MAstV-17	0.78
EU847144 BaAstV AFCD57 MAstV-14	0.78	EU847144 BaAstV AFCD57 MAstV-14	0.78
FJ571066 BaAstV LD77 MAstV-15	0.78	FJ571066 BaAstV LD77 MAstV-15	0.78
FJ571071 BaAstV DX19 MAstV-19	0.79	FJ571071 BaAstV DX19 MAstV-19	0.79
FJ973620 HuAstV VA1 MAstV-9	0.79	FJ973620 HuAstV VA1 MAstV-9	0.79
EU847145 BaAstV AFCD11 MAstV-16	0.79	GQ502193 HuAstV VA2 WD0680 MAstV-8	0.79
GQ502193 HuAstV VA2 WD0680 MAstV-8	0.79	EU847145 BaAstV AFCD11 MAstV-16	0.79
KF233994 BoAstV NeuroS1 MAstV-13	0.79	KM035759 BoAstV CH13 MAstV-13	0.79
KM035759 BoAstV CH13 MAstV-13	0.79	KF233994 BoAstV NeuroS1 MAstV-13	0.79
AY179509 MiAstV MAstV-10	0.80	AY179509 MiAstV MAstV-10	0.80
FJ890355 BdAstV Bd1 MAstV-7	0.80	JF755422 MoAstV M-52 MAstV-20	0.80
JF755422 MoAstV M-52 MAstV-20	0.80	FJ890355 BdAstV Bd1 MAstV-7	0.80
FJ571067 BaAstV LD71 MAstV-12	0.80	FJ571067 BaAstV LD71 MAstV-12	0.80
Y15937 OvAstV Snodgrass MAstV-13	0.80	Y15937 OvAstV Snodgrass MAstV-13	0.80
GU985458 MiAstV SMS MAstV-21	0.80	GU985458 MiAstV SMS MAstV-21	0.80
KT956903 BoAstV CH15 MAstV-13	0.80	EU847155 BaAstV AFCD337 MAstV-18	0.80
LN879482 BoAstV BH89/14 MAstV-13	0.80	KT956903 BoAstV CH15 MAstV-13	0.80
EU847155 BaAstV AFCD337 MAstV-18	0.81	LN879482 BoAstV BH89/14 MAstV-13	0.80

4. Discussion

Enteric infection with animal and human AstVs have been associated with diarrhea [16], however the role of BoAstV as a causative agent of diarrhea in cattle is controversial and has not been extensively studied. Although BoAstV has been isolated from diarrheic calves [1], initial attempts to experimentally reproduce the disease have been unsuccessful [2], and, in addition, neonatal calf diarrhea generally has a multifactorial origin; the aim of our study was not to establish an association between the presence of the virus and disease, but to determine the prevalence and genetic diversity of BoAstV circulating in dairy herds from Uruguay. The present survey showed a high prevalence of infection with a broad diversity of BoAstV. However, because there is great genetic diversity within the MAstV genus, and this may have implications in pathogenicity [17], further studies are needed to assess whether particular species in this genus are associated with enteric disease in cattle, as seems to be the case for bovine encephalitis linked to MAstV-13 [3,4]. To date, most BoAstVs are not assigned to any of the 19 MAstV species confirmed by the ICTV [9]. Nevertheless, recent studies indicate that BoAstV can be tentatively assigned to the species MAstV-13, MAstV-24, MAstV-28, MAstV-29, MAstV-30 and MAstV-33 [10].

The 432-nucleotide fragment of the polymerase gene of AstV [13] has been widely used for the screening of BoAstV throughout the world, both in fecal and central nervous system samples [11,18–20]; the RT-PCR has become a common tool for the detection of astrovirus because of its higher sensitivity compared with other techniques [21].

The phylogenetic diversity of Uruguayan BoAstV strains analyzed using the partial polymerase region was high, with the detection of 3/25 (12%) MAstV-28, 3/25 (12%) MAstV-33, and 19/25 (76%) of an unclassified MAstV species. BoAstV strains detected in Uruguay were closely related to BoAstVs detected in Brazil [18]. Several studies have classified BoAstVs in lineages based on the polymerase and/or capsid partial sequences [18,19,22–24]; most of the strains detected in these works clustered with the unclassified Uruguayan strains, indicating that this species is widely distributed worldwide (being probably the most prevalent) and demonstrating the importance of its correct classification.

Complete amino acid capsid sequence analyses are mandatory to establish new MAstV species [9]. In order to attempt to classify the divergent Uruguayan strains, capsid sequences were obtained. Unfortunately, probably due to the high variability in this genomic region, six partial sequences and only two complete capsid sequences were obtained. The phylogenetic analyses with the complete capsid sequences of the Uruguayan strains LVMS681 and LVMS2704 confirmed the results obtained with the polymerase region. Both strains clustered in a monophyletic group with other unclassified strains, both at the amino acid and the nucleotide level. These results were confirmed by the analysis of genetic differences in the complete capsid amino acid sequence, suggesting that the unclassified MAstV species found in this study should be assigned to a new MAstV species, as proposed by the ICTV.

The tentatively assigned species MAstV-13, MAstV-24, MAstV-29, MAstV-30, MAstV-33 and the unclassified MAstV species have p-distances within groups lower than 0.35, and between groups higher than this cut-off value determined for the standardized classification criteria [9] (Table 2). Surprisingly, MAstV-28 showed controversial results within this species, with p-distances at the amino acid level among strains higher than 0.350 (except between LC047790 and LC047798), the cut-off value for the species classification criteria. It is documented that, at initial stages of a viral emergence, sequences are separated almost exclusively by transient polymorphisms and not by fixed differences [25], and mutations accumulate more rapidly [26,27]. Probably, this higher genetic variation is due to incomplete purifying selection and some slightly deleterious mutations fall on external branches of phylogeny [26,27]. Some strains belonging to the MAstV-28 species were detected recently in nasopharyngeal samples [5], suggesting that these strains are adapting to tissues beyond the gastrointestinal tract [28]. Additional information of the strain LVMS1376 (from which the capsid region was partially amplified and classified as MAstV-28) was detected in a 3-month-old Holstein calf, which presumably died of pneumonia, although this data should be considered with caution, since the BoAstV detection and characterization was conducted from the intestinal content of this animal. The phylogenetic analysis revealed that MAstV-28 probably has a common ancestor with

buffalo astrovirus, as previously described for other MAstV species [11], as well as ovine–bovine or porcine–bovine interspecies transmission events [23]. However, further studies are needed to better understand the molecular epidemiology of MAstVs.

The strain LVMS2692 showed controversial results; it clustered with the MAstV-unclassified strains in the polymerase genomic region used for screening, and with the MAstV-29 strain in the partial capsid region, suggesting coinfection and/or recombination. The region comprised between these two regions was amplified with primers BoAstV-F and BoAstV-CAP-U-33R1, but, unfortunately, chromatograms of both DNA strands showed mixed populations and could not be read, probably due to coinfection with the unclassified MAstV and MAstV-29. Coinfection has been described in BoAstV [13], and could be underestimated.

As the region of basic amino acids at the N-terminus of the capsid protein is thought to interact with the genomic RNA inside the virion [29], additional studies should be done to determine the implications of the insertion observed in the Uruguayan strains belonging to the unclassified MAstV.

Evidence indicates that the BoAstVs that belong to MAstV-13 are associated with neurological diseases and encephalitis [3,4,20], while the other species are not clearly associated to disease, but are excreted in feces [11,13,18,22,23], and/or detected in nasopharyngeal exudates [5]. On the other hand, it is important to study the consequence of asymptomatic cases in the epidemiology and transmission of the virus [17]. Interestingly, most of the strains from throughout the world [17,18,20–22] that could belong to the MAstV characterized in this work were mainly detected in diarrheic samples, although the role of BoAstV in the development of diarrhea in cattle needs further clarification.

5. Conclusions

BoAstV was highly prevalent in dairy calves in Uruguay; there was a high genetic diversity between strains and a MAstV species was characterized and classified. In addition, our analyses suggest that this species is the most prevalent worldwide, reinforcing the need for it to be correctly classified. Further studies are necessary to elucidate the possible enteropathogenicity of BoAstV species in cattle.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/1/32/s1. Figure S1: Maximum-likelihood tree constructed with complete capsid nucleotide sequences of MAstVs, Figure S2: Maximum-likelihood tree constructed with partial capsid amino acid sequences, Table S1: Partial polymerase sequences used for the phylogenetic analysis, and Table S2: Complete capsid sequences used for the MAstV species classification.

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Artículo 6. El primer caso de encefalitis asociada a astrovirus bovino en el hemisferio sur (Uruguay), revela evidencia de introducción viral a las Américas desde Europa

Este artículo está relacionado con el objetivo específico 3.2.1., orientado a desarrollar metodologías moleculares para la detección y caracterización molecular de los virus, incluido BoAstV. Si bien no se relaciona con la DNT, fue un estudio oportunista, aprovechando la metodología puesta a punto para detectar BoAstV en materia fecal y contenido intestinal, extendiéndola a otro tipo de muestra, en este caso cerebro. Se trató de un caso de encefalitis con posible causa viral, que llegó a nuestro laboratorio para analizarlo para Herpesvirus bovino tipo 1 y 5, obteniendo resultado negativo, al igual que para otros posibles patógenos analizados en otros laboratorios.

Como BoAstV es un virus emergente en cuanto a encefalitis, y debido a que contábamos con la metodología para detectarlo, se analizó, obteniendo un resultado positivo. Se trató del primer caso de encefalitis asociada a astrovirus a nivel de todo el hemisferio sur, siendo que hasta el momento solamente se habían confirmado casos tanto en humanos como en otros animales a nivel del hemisferio norte (América del Norte, Europa, Asia). Además de confirmar a BoAstV como la causa de la encefalitis (y muerte del novillo), se logró determinar que el virus llegó a América desde Europa hace unos 100 años.

Este trabajo, dio pie a iniciar una nueva línea de investigación, en astrovirus emergentes en bovinos, detectando un segundo caso de astrovirus asociado a encefalitis en una vaca (Encefalitis asociada a astrovirus bovino neurotrópico, ¿una enfermedad subdiagnosticada en Sudamérica?), así como también algunos casos de detección de astrovirus posiblemente asociado a casos de neumonía, los cuales están siendo investigados.

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The First Case of Bovine Astrovirus-Associated Encephalitis in the Southern Hemisphere (Uruguay), Uncovers Evidence of Viral Introduction to the Americas From Europe

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Astrovirus species members of the *Mamastrovirus* genus (family *Astroviridae*) have been increasingly recognized as neuroinvasive pathogens in various mammals, including humans, mink, cattle, sheep, and pigs. While cases of astrovirus-associated encephalitis have been reported in North America, Europe, and Asia, their presence has never been documented in the Southern hemisphere. This paper describes a case of astrovirus-associated encephalitis in cattle in Uruguay that broadens the geographic distribution and genetic diversity of neuroinvasive astroviruses and provides phylogeographic evidence of viral introduction to the Americas from Europe. A 22-month-old Holstein steer from a farm in Colonia Department, Uruguay developed progressive neurological signs over a 3-days period before dying. Histopathological examination of the brain and proximal cervical spinal cord revealed disseminated, moderate to severe lymphocytic, histiocytic, and plasmacytic poliomeningoencephalomyelitis with neuronal necrosis. A *Mamastrovirus* strain in the CH13/NeuroS1 clade, that we called bovine astrovirus (BoAstV)-Neuro-Uy, was identified by reverse transcriptase PCR followed by nearly complete genome sequencing. Additionally, BoAstV was detected intralesionally in the brain by chromogenic RNA *in situ* hybridization within neuronal perikarya, axons and dendrites. Phylogenetic analysis of BoAstV-Neuro-Uy revealed a close relationship to neurotropic BoAstVs within the Virginia/Human-Mink-Ovine clade, which contains a growing cadre of neuroinvasive astroviruses. Analyzing the complete coding region of neuroinvasive BoAstVs sequences available in GenBank, we estimated an evolutionary

rate of 4.27 × 10^{-4} (95% HPD 2.19–6.46 × 10^{-4}) nucleotide substitutions/site/year. Phylogeographic analysis suggests that the common viral ancestor circulated in Europe between 1794–1940, and was introduced in Uruguay between 1849–1967, to later spread to North America and Japan.

Keywords: bovine astrovirus, cattle, encephalitis, infectious diseases, *Mamastrovirus*, phylogeography, South America, Uruguay

INTRODUCTION

The *Astroviridae* family contains non-enveloped, positive-sense, single-stranded RNA viruses within two genera, *Mamastrovirus* and *Avastrovirus*, which infect mammals and birds, respectively. Currently, the International Committee on Taxonomy of Viruses (International Committee on Taxonomy of Viruses [ICTV], 2018) recognizes 19 species, namely *Mamastrovirus-1* through *-19*, within the *Mamastrovirus* genus; however, there are numerous strains awaiting classification, some of which are tentatively considered new species (Donato and Vijaykrishna, 2017).

Since 2010, several astroviruses have increasingly been recognized as neuroinvasive in various mammalian species, including humans (Quan et al., 2010; Naccache et al., 2015), mink (Blomström et al., 2010), cattle (Li et al., 2013), sheep (Pfaff et al., 2017), and pigs (Boros et al., 2017). After initial recognition of bovine astrovirus-associated encephalitis in United States cattle (Li et al., 2013), a retrospective study in cases of sporadic bovine encephalitis of undetermined etiology from Switzerland revealed that this neuroinvasive astrovirus had gone undetected for decades (Selimovic-Hamza et al., 2016). Although the epidemiology and transmission routes of these astroviruses are unknown, cross-species transmission has been suggested based on the high level of identity (>98%), shared between bovine and ovine neuroinvasive astroviruses at the nucleotide and amino acid levels (Boujon et al., 2017).

Bovine astroviruses (BoAstVs), named BoAstV-NeuroS1 (Li et al., 2013) and BoAstV-CH13 (Bouzalas et al., 2014), were initially found in the brain of cattle with non-suppurative encephalitis in the United States and Switzerland, respectively. Despite the different nomenclature, both viruses represent the same genotype species (Bouzalas et al., 2016; Selimovic-Hamza et al., 2017a) that is still awaiting official classification by the ICTV. In 2015, a previously unknown BoAstV strain, named BoAstV-CH15, was identified in the brain of cows with encephalitis in Switzerland. Full genome phylogenetic comparison revealed a closer relationship of BoAstV-CH15 with an ovine astrovirus (OvAstV) than with BoAstV-CH13 (Seuberlich et al., 2016). Coinfection with BoAstV-CH13 and BoAstV-CH15 was also documented in one case (Seuberlich et al., 2016). The same year in Germany, Schlottau et al. (2016) reported a novel astrovirus, namely BoAstV-BH89/14, in a cow with encephalitis, that was most closely related to OvAstV and BoAstV-CH15. Subsequently, BoAstV-CH13/NeuroS1 was identified in 2017 in cases of bovine encephalitis in eastern and western Canada (Spinato et al., 2017; Selimovic-Hamza et al., 2017b). In 2018, a novel neuroinvasive BoAstV closely related with North American and European BoAstV-NeuroS1/BoAstV-CH13, was identified in a steer with non-suppurative encephalomyelitis in Japan, and the occurrence of intra-genotypic recombination between the North American and European strains was suggested (Hirashima et al., 2018).

While cases of astrovirus-associated encephalitis have been reported in North America, Europe, and Asia, their presence has never been documented in the Southern hemisphere. Here we describe a case of astrovirus-associated encephalitis in cattle in Uruguay, which broadens the geographic distribution and genetic diversity of neuroinvasive astroviruses and provide phylogeographic evidence that suggests that this virus was introduced into the Americas from Europe and later spread to Asia.

MATERIALS AND METHODS

History and Signalment

In June 2018, a 22-month-old Holstein steer in a group of 37 steers in a ∼300-hectare farm in Colonia, Uruguay, developed progressive neurological sings including unusual behavior, aimless walking, circling, ataxia, repetitive and uncoordinated tongue movements, and recumbency. The herd grazed on an annual oat pasture and was supplemented with corn silage. A presumptive clinical diagnosis of cerebral listeriosis by the veterinary practitioner prompted treatment with penicillin and streptomycin, however the animal died spontaneously after a clinical course lasting 3 days.

Pathologic Examination, *in situ* Hybridization (ISH) and Immunohistochemistry (IHC)

The head of the steer was removed from the carcass and submitted to INIA's Veterinary Diagnostic Laboratory (Animal Health Platform) for diagnostic work-up. Half of the brain, a short segment of proximal cervical spinal cord (C1), trigeminal ganglion and root of the trigeminal nerve, salivary gland, retropharyngeal lymph node, oropharynx, esophagus, tongue and skeletal muscle, were immersion-fixed in 10% neutral buffered formalin for 48–72 h. Tissues were routinely processed for histology, embedded in paraffin, microtome-sectioned at 4–5 μm and stained with hematoxylin and eosin (H&E) and Gram stains.

Chromogenic ISH was performed manually on 5 μm sections of formalin-fixed, paraffin-embedded (FFPE) brainstem, cerebrum and cerebellum on Superfrost Plus slides (Thermo Fisher Scientific, Pittsburgh, PA, United States) using the

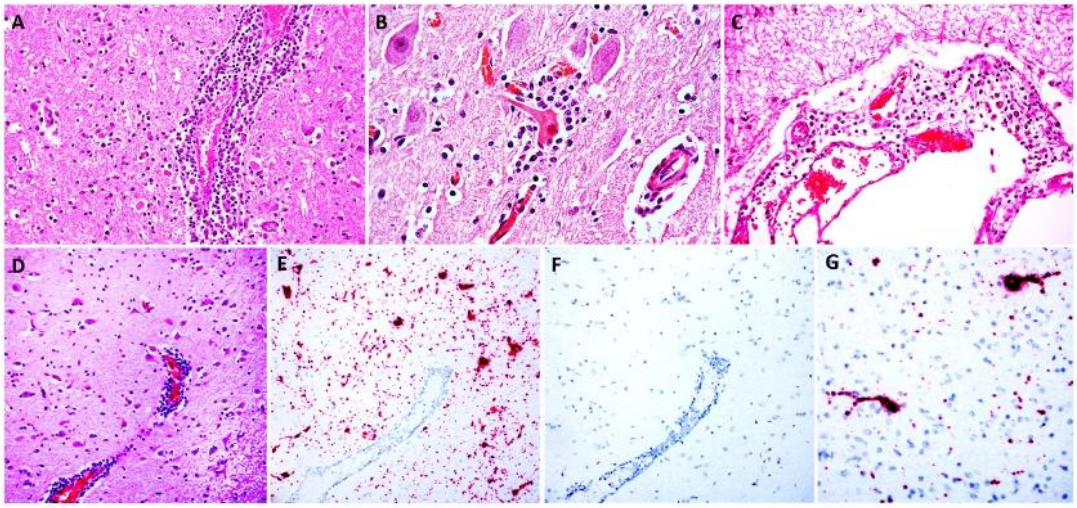

FIGURE 1 | Histologic lesions in the brainstem **(A,B)** and cerebral cortex **(C,D)** and detection of BoAstV RNA in the cerebral cortex **(E,G)**. Images **(A–D)** are sections of brain stained with H&E; images E and G are sections of cerebral cortex demonstrating hybridization using chromogenic ISH using the BoAstV specific probe, counterstained with hematoxylin; image F is a serial section of cerebral cortex DapB probe (negative control), counterstained with hematoxylin. **(A)** A perivenular space is markedly expanded by inflammatory cells (mostly lymphocytes and histiocytes) that also infiltrate the adjacent neuropil. **(B)** The neuron in the center has hypereosinophilic perikaryon and karyorrhexis (necrosis) and the neuronal body is surrounded by increased numbers of glial (satellitosis) and inflammatory cells. **(C)** The leptomeninge is infiltrated by lymphocytes and histiocytes. **(D)** A region of cerebral cortex with multiple hypereosinophilic (necrotic) neurons and a large vessel with perivascular lymphocytic cuffs. **(E)** In a serial section of cerebral cortex, the abundant intracytoplasmic BoAstV RNA labeling is depicted by strong, granular red chromogen deposition within neuronal cytoplasm of the soma and neuronal extensions **(E,G)**, that is not present with hybridization using the negative control probe **(F)**.

RNAscope 2.5 Red assay kit (Cat #322360, Advanced Cell Diagnostics, Hayward, CA, United States) and the BoAstV probe Cat. #406921. The probe is composed of 20ZZ pairs targeting region 5232–6180 of the virus (GenBank KF233994.1). Each 5 μm section of tissue was pretreated with heat and protease prior to probe hybridization for 2 h at 40°C and processed as per the manufacturers' recommendations. Negative controls used for validation of signal included an unrelated (GC-content matched) probe run on serial sections and probing tissue from uninfected animals. Slides were counterstained with hematoxylin and mounted with EcoMount (Biocare Medical, Concord, CA, United States).

Additionally, IHC was performed in FFPE sections of brainstem, cerebrum and cerebellum, as previously described, for the identification of West Nile virus (WNV, *Flavivirus*) (Palmieri et al., 2011), rabies virus (*Lyssavirus*) (Stein et al., 2010), and *Chlamydia* spp. (Giannitti et al., 2016) antigens.

Molecular Virology

Nucleic acid extraction was accomplished from a pooled sample of frozen (−20°C) brain using MagMAX Nucleic Acid Isolation Kit® (Thermo Fisher Scientific). For astrovirus detection, reverse transcription (RT) was performed with RevertAid Reverse Transcriptase® (Thermo Fisher Scientific) and random hexamer primers (Qiagen). PCR was performed from cDNA using MangoMix® (Bioline) and primers that amplify a 432-nucleotide fragment of the astrovirus polymerase gene (Tse et al., 2011). The PCR product was visualized in 2% agarose gel, purified using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen), and sequenced at Macrogen Inc. (Seoul, South Korea). For astrovirus whole genome amplification, Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) and oligo(dT)18 for obtention of cDNA, and MangoMix® (Bioline) or Ranger DNA Polymerase (Bioline) with primers described by Hirashima et al., 2018, were used. The PCR products were visualized in 1–2% agarose gel, purified and sequenced as mentioned above. Sequence assembly was conducted with SeqMan (Lasergene 8, DNASTAR). Twenty-six complete genome sequences of neuroinvasive astrovirus from cattle, sheep, pigs, humans and mink, and enteric bovine astrovirus available in GenBank were downloaded and aligned using Clustal W in MEGA 7 software (Kumar et al., 2016). W-IQ-TREE[1] (Trifinopoulos et al., 2016) was used to determine the best-fit model of sequence evolution (SYM+I+G4) and to construct a maximum-likelihood phylogenetic tree with the nearly complete sequences of the BoAstV detected in this case, and those complete sequences downloaded from GenBank, using bootstrap as the statistical method to assess clades robustness. Similarity plot was performed with SimPlot software (Lole et al., 1999). *P*-distances at amino acid level of the ORF2 were estimated with MEGA 7 software (Kumar et al., 2016).

Additionally, a Bayesian phylogeographic analysis was performed with the BEAST v1.8.4 package (Drummond et al., 2012), using: the complete coding region of BoAstV CH13/NeuroS1 lineage, ORF1ab (non-structural genes), ORF2 (structural gene), ORF1a (protease) and a partial ORF1b

[1] http://iqtree.cibiv.univie.ac.at

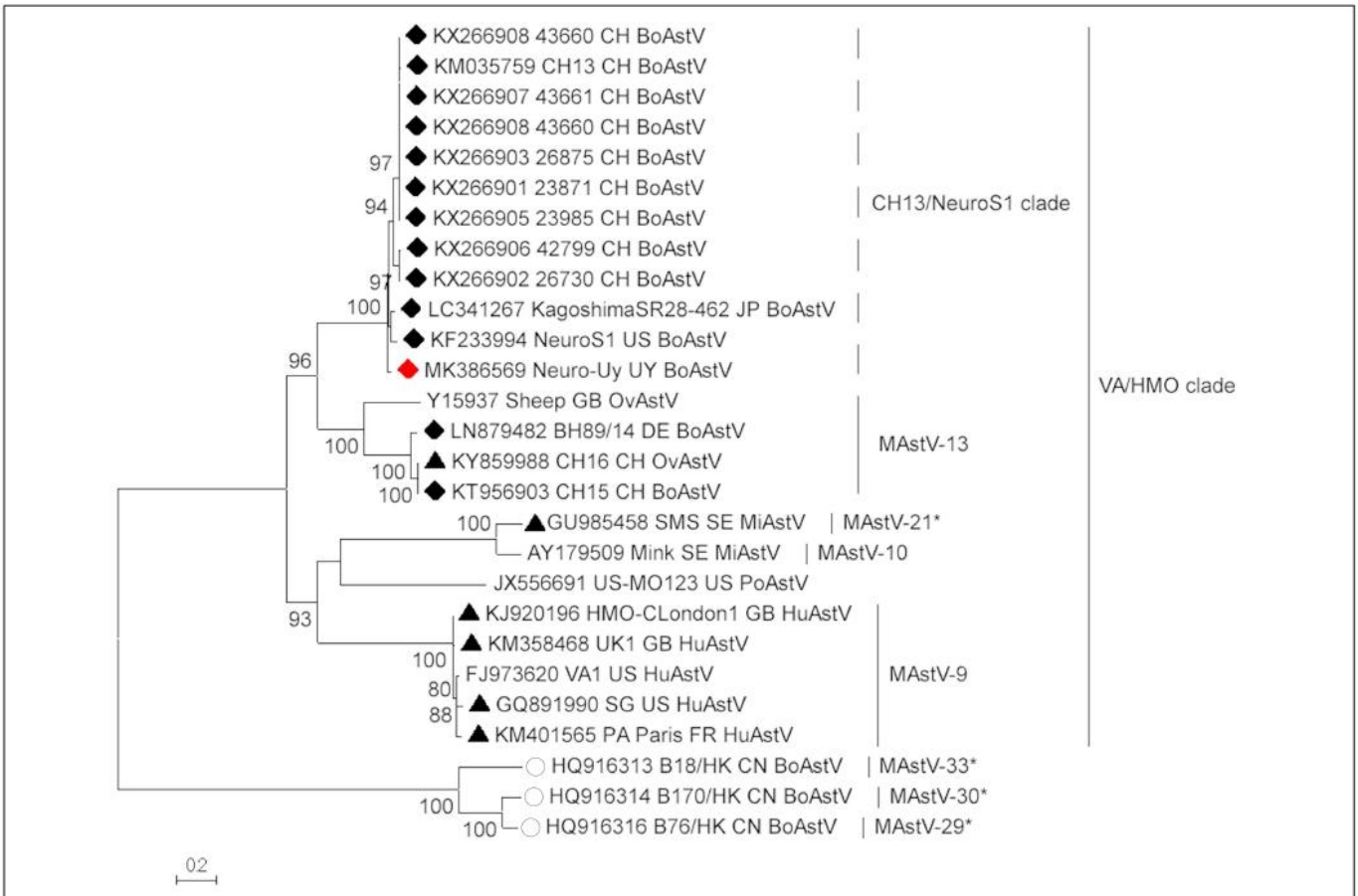

FIGURE 2 | Phylogenetic analysis of full-length nucleotide sequences by maximum-likelihood method. BoAstV-Neuro-Uy is marked with a red rhombus. Other sequences of bovine neuroinvasive astroviruses are marked with black rhombi, sequences from non-bovine neuroinvasive astroviruses are marked with black triangles, and sequences from enteric bovine astroviruses are marked with white circles. *Mamastrovirus* species are shown; asterisks indicate species yet to be recognized by the ICTV. Strains within the CH13/NeuroS1 clade not yet assigned a species by the ICTV are identified with a dashed line. Bootstrap values are indicated in nodes. CH, Switzerland; JP, Japan; US, United States of America; UY, Uruguay; GB, United Kingdom of Great Britain; DE, Germany; SE, Sweden; FR, France; CN, China. BoAstV, bovine astrovirus; OvAstV, ovine astrovirus; PoAstV, porcine astrovirus; HuAstV, human astrovirus; MiAstV, mink astrovirus.

(polymerase genomic region, for which Canadian strains were available), with all the sequences available in GenBank (last accession April 18, 2019), to determine the evolutionary rate, the ages/years of the common ancestors, and the most probable route of viral circulation by country (Switzerland, Uruguay, United States, Canada, and Japan). Lack of recombination in the dataset was determined using Recombination Detection Program 4. The substitution model that best fit each alignment was determined using MEGA 7 software through Bayesian information criterion (BIC) values, and the temporal structure of each dataset was evaluated using TempEst (Rambaut et al., 2016). The lognormal relaxed molecular clock with Bayesian Skyline analysis was selected by Bayes Factor among the different combinations of molecular clocks and coalescent tree priors used. The country of detection was used as trait. The Markov chain Monte Carlo length was 100 million generations, ensuring the convergence of the analysis, evaluated in Tracer v1.6.0, and the posterior probability was used to evaluate clades. The maximum clade credibility tree (MCCT) was obtained using TreeAnnotator software from BEAST and visualized in FigTree v1.4.3.

Lastly, DNA extracted from frozen brain was processed by PCR for the detection of bovine herpesviruses 1 and 5 (BHV-1 and -5), as previously described (Ashbaugh et al., 1997).

Bacteriology

Fresh samples of cerebrum and brainstem were routinely processed for aerobic bacterial cultures in blood and MacConkey agars, and selective culture for *Listeria monocytogenes* (Al-Zoreky and Sandine, 1990).

RESULTS AND DISCUSSION

The clinical signs and epidemiological findings in the case described herein, albeit non-specific, were similar to those described in other cases of bovine astrovirus-associated encephalitis, which is usually described as sporadic (Selimovic-Hamza et al., 2016), with a variety of neurological deficits (Deiss et al., 2017), with a duration of clinical signs that typically ranges from 1 day to 3 weeks

TABLE 1 | Estimates of evolutionary divergence at the amino acid level of the complete ORF2 region between sequences of bovine and ovine *Mamastrovirus-13* and strains within the CH13/NeuroS1 clade not yet assigned to a species by the International Committee on Taxonomy of Viruses (ICTV).

Sequences and GenBank accession numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1- KY859988_OvAstV_CH16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2- Y15937_OvAstV_OvAstV-1	0.263	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3- LC341267_BoAstV_KagoshimaSR28-462	0.343	0.317	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4- KM035759_BoAstV_CH13	0.341	0.321	0.012	–	–	–	–	–	–	–	–	–	–	–	–	–
5- KF233994__BoAstV_NeuroS1	0.348	0.327	0.016	0.015	–	–	–	–	–	–	–	–	–	–	–	–
6- KX266902_BoAstV_26730	0.341	0.321	0.012	0.005	0.015	–	–	–	–	–	–	–	–	–	–	–
7- KX266903_BoAstV_26875	0.344	0.320	0.012	0.005	0.015	0.005	–	–	–	–	–	–	–	–	–	–
8- KX266907_BoAstV_43661	0.341	0.320	0.009	0.003	0.012	0.003	0.003	–	–	–	–	–	–	–	–	–
9- KX266901_BoAstV_23871	0.343	0.321	0.012	0.005	0.012	0.005	0.005	0.003	–	–	–	–	–	–	–	–
10- KX266906_BoAstV_42799	0.341	0.320	0.011	0.004	0.013	0.004	0.004	0.001	0.004	–	–	–	–	–	–	–
11- KX266908_BoAstV_43660	0.341	0.320	0.011	0.004	0.013	0.004	0.004	0.001	0.004	0.003	–	–	–	–	–	–
12- KX266905_BoAstV_23985	0.341	0.318	0.012	0.005	0.015	0.005	0.005	0.003	0.005	0.004	0.004	–	–	–	–	–
13- KX266904_BoAstV_36716	0.341	0.320	0.008	0.004	0.013	0.004	0.004	0.001	0.004	0.003	0.003	0.004	–	–	–	–
14- MK386569_BoAstV_Neuro-Uy	0.342	0.323	0.016	0.013	0.019	0.013	0.013	0.011	0.013	0.012	0.012	0.013	0.012	–	–	–
15- KT956903_BoAstV_CH15	0.007	0.266	0.347	0.345	0.352	0.345	0.347	0.345	0.347	0.345	0.345	0.345	0.345	0.346	–	–
16- LN879482_BoAstV_BH89/14	0.007	0.260	0.344	0.343	0.349	0.343	0.344	0.343	0.344	0.343	0.343	0.343	0.343	0.344	0.011	–

Based on current ICTV criteria, strains with p-distances < 0.35 should be considered within the same genotype species. The number of amino acid differences per site between sequences are shown. The analysis involved 16 amino acid sequences. All ambiguous positions were removed for each sequence pair. There was a total of 773 positions in the final dataset. OvAstV, ovine astrovirus; BoAstV, bovine astrovirus.

(Schlottau et al., 2016; Deiss et al., 2017; Spinato et al., 2017; Hirashima et al., 2018).

Macroscopic examination of the brain, the C1 segment of the spinal cord, and other tissues of the head did not reveal significant gross anatomic lesions. Histologically, there was moderate to severe, lymphocytic, histiocytic and plasmacytic meningoencephalomyelitis affecting the telencephalon (including the cerebral hemisphere and hippocampus), brainstem, and the only examined segment of spinal cord. Lesions were predominantly distributed in the gray matter and limiting areas of white matter. In affected areas there was perivascular cuffing and lymphoplasmacytic and histiocytic inflammation and neuronal necrosis/neuronophagia with gliosis in the adjacent neuropil. There was satellitosis of affected, necrotic neurons (**Figures 1A–D**). The lesions were much less frequent and severe in the cerebellar parenchyma, although there was multifocal moderate cerebellar leptomeningitis. No intralesional bacteria were found with H&E and Gram stains. No significant histologic changes were found in the other examined tissues.

A neuroinvasive viral infection was suspected upon histologic examination of the central nervous system. Cattle with encephalitis are of concern because many ruminant neuropathogens are zoonotic (Cantile and Youssef, 2016); thus, a diagnosis of encephalitis should prompt extensive laboratory testing to screen for infectious agents when possible. In the case described herein, IHCs for WNV, rabies virus and *Chlamydia* spp., and PCR for BHV-1 and -5 were all negative, and no pathogenic bacteria were cultured from brain tissue. Because the steer was < 2 years old and no spongiform changes were observed in the brainstem, the animal was not tested for bovine spongiform encephalopathy (BSE), which is an exotic disease of adult cattle that has never been reported in Uruguay. Moreover, BSE is not inflammatory (Cantile and Youssef, 2016).

In situ hybridization was performed using a probe generated from BoAstV-NeuroS1, and there was probe hybridization abundant within, and limited to the cytoplasm of neurons in the cerebral hemisphere and hippocampus (**Figures 1E–G**). In these areas the probe hybridization colocalized with necrotic neurons and regions of gliosis, with no probe hybridization detectable in the glial cells, or inflammatory cells of the perivascular cuffs. No viral nucleic acid was detected by ISH in the cerebellum, which had only minimal inflammatory lesions in the parenchyma but moderate leptomeningitis, or the brainstem, including sections with severe inflammation. This means that, topographically, detection of viral distribution by ISH was more limited than encephalitis in the sections examined, which has occasionally been described in cases of BoAstV-CH13/NeuroS1-associated encephalitis in cattle (Selimovic-Hamza et al., 2017a,b). A reason for this occasional lack of viral RNA detection in lesioned areas of brain might be the detection limit of the ISH, or the clearance of the virus in inflamed areas of the brain by the time of death, as previously suggested (Selimovic-Hamza et al., 2017b). As expected, no probe hybridization was detected by ISH in the brain tissue used as negative control.

Astrovirus was detected in brain by RT-PCR. Nearly complete genome sequence analysis revealed a *Mamastrovirus* strain within

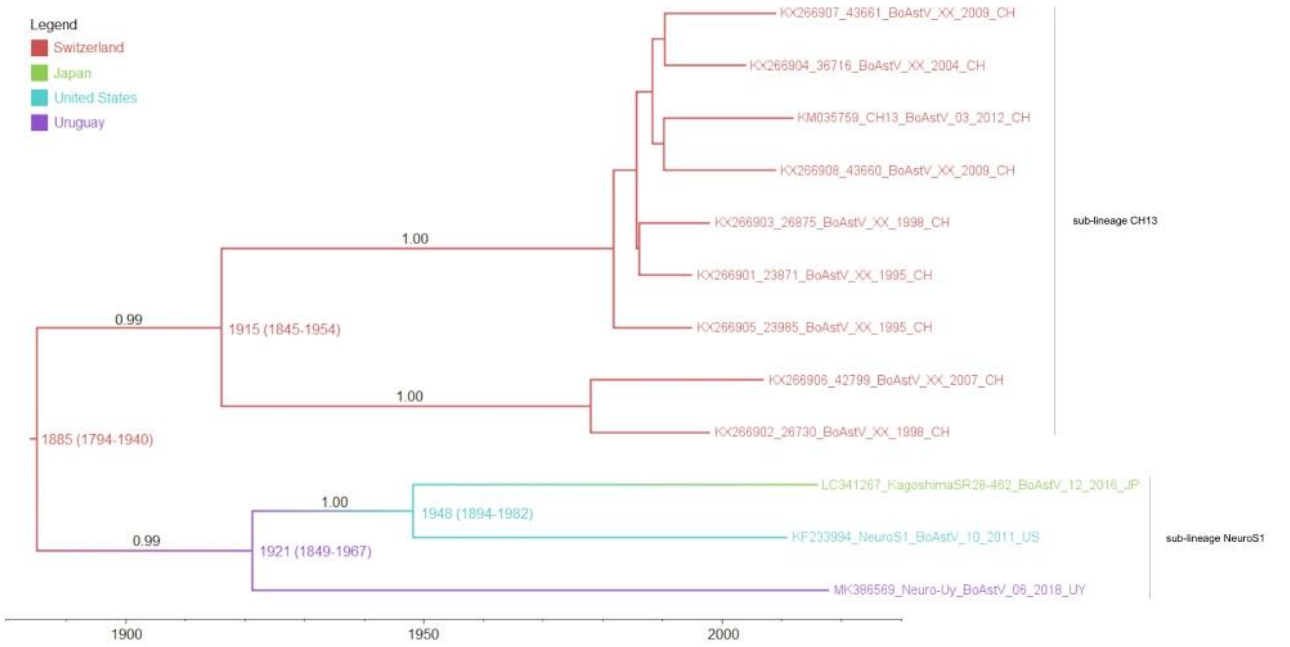

FIGURE 3 | Maximum clade credibility tree obtained by analysis of the full-length coding region. The color of the branches represents the most likely country where the ancestors circulated, posterior probability values are shown in the branches and the numbers in each node represent the years of origin for each clade with the 95% HPD interval. Sub-lineages are indicated with labels.

the CH13/NeuroS1 clade, we named BoAstV-Neuro-Uy, the sequence was deposited in GenBank under accession number MK386569. The phylogenetic analysis revealed proximity with other neuroinvasive astroviruses within the Virginia/Human-Mink-Ovine (VA/HMO) clade (**Figure 2**), which contains most known neuroinvasive astroviruses (Hirashima et al., 2018; Reuter et al., 2018). The almost complete sequence of BoAstV-Neuro-Uy is 6427 bp in length and has a sequence identity of 94% with KagoshimaSR28-462 strain. BoAstV-Neuro-Uy has similar features as other strains of lineage CH13/NeuroS1: a 5′UTR region of 51 nt, ORF1a (protease) of 861 amino acids (aa), ORF1b of 523 aa (RNA-dependent RNA polymerase), and ORF2 of 758 aa (capsid protein). Unfortunately, the 3′UTR could not be sequenced, but a poly(A) tail is presumed to be present because oligo(dT)18 was used to obtain cDNA. In addition, the heptameric AAAAAAC sequence, a ribosomal frameshift signal, is present. *P*-distances at the amino acid level of the ORF2 confirmed the assignation of this strain to the CH13/NeuroS1 clade. *P*-distances < 0.35 between BoAstV-Neuro-Uy and other members of this clade (**Table 1**) would support a classification of these viral strains within one same species; *Mamastrovirus-13* has been recently proposed by other authors (Donato and Vijaykrishna, 2017; Hirashima et al., 2018), although definite species assignation by the ICTV is pending. The probe used for the ISH, generated from BoAstV-NeuroS1, had 92.7% sequence identity with BoAstV-Neuro-Uy.

Studies based on neuropathological examinations and astrovirus nucleic acid and protein detection have concluded that there is a probable causal relationship between astrovirus infection and neurological disease and lesions in cattle (Selimovic-Hamza et al., 2017a; Reuter et al., 2018). To the best of our knowledge astrovirus-associated encephalitis has not been reproduced experimentally yet. This would require isolation of neuroinvasive astroviruses from clinical cases, which was not attempted in our case.

The source of BoAstV-Neuro-Uy in this case could not be determined. However, reservoir cattle and wildlife should be considered, as the cattle were raised under extensive outdoor conditions. The affected animal had been purchased and moved to the farm in February 2018 along with other 9 steers. Unfortunately, the owner refused further sampling and testing of other animals in the property, and a more detailed epidemiological investigation. None of the other animals in the group had developed neurological disease as of August 2018, the last time the veterinary practitioner was contacted. A seasonality from the beginning of winter until the end of the spring has been suggested for cases of astrovirus-associated encephalitis in Switzerland (Selimovic-Hamza et al., 2016). Interestingly, the case described herein occurred in June, corresponding to the autumn-winter transitional period in the Southern hemisphere.

While neurotropic astroviruses have been identified in North America (Li et al., 2013; Spinato et al., 2017), Europe (Bouzalas et al., 2014) and Asia (Hirashima et al., 2018), their presence has never been reported in the Southern hemisphere, so this communication broadens the geographic distribution of astrovirus-associated encephalitis. To assess whether the viral strain detected in Uruguay might have originated in Europe, North America, or Asia, we estimated the evolutionary rate and performed a phylogeographic analysis using neuroinvasive BoAstV sequences available in GenBank. The evolutionary rate

estimated using the complete coding region was 4.27×10^{-4} (95% highest probability density -HPD-, $2.19–6.46 \times 10^{-4}$) nucleotide substitutions/site/year, which is expected for an RNA virus (Jenkins et al., 2002), but lower than that estimated for enteric human astroviruses (Babkin et al., 2012, 2014). The ORF1ab region showed a similar evolutionary rate (4.20×10^{-4}, 95% HPD $1.66–6.46 \times 10^{-4}$ substitutions/site/year) as the complete coding region, while the ORF1a (2.92×10^{-4}, 95% HPD $1.19 \times 10^{-6}–6.46 \times 10^{-4}$ substitutions/site/year) and ORF2 (2.86×10^{-4}, 95% HPD $4.13 \times 10^{-6}–5.79 \times 10^{-4}$ substitutions/site/year) showed a slightly faster evolutionary rate, and the partial polymerase genomic region (ORF1b) showed a slightly slower evolutionary rate (5.39×10^{-4}, 95% HPD $6.41 \times 10^{-7}–1.10 \times 10^{-3}$ substitutions/site/year).

As determined by the phylogeographic analysis with the complete coding region, and shown in the MCTT (**Figure 3**), there are two sub-lineages (CH13 and NeuroS1) based on reference strains, that have a common ancestor. The most recent common ancestor of these sub-lineages (lineage CH13/NeuroS1) arose in Europe approximately in 1885 (95% HPD, 1794–1940). At the beginning of the 1900's, the two sub-lineages diverged, the CH13 sub-lineage stayed circulating in Europe, while the NeuroS1 sub-lineage spread to America and Asia. The most likely scenario is that the NeuroS1 sub-lineage was introduced in Uruguay from Europe around the year 1921 (95% HPD, 1849–1967), presumably through livestock trade, then spread to North America, and later to Japan (**Figure 3**). Due to the limitation in the number of sequences available in GenBank, which could have biased the analysis, the results obtained using the complete coding region were compared with those obtained with other genomic regions (ORF1ab, ORF2, ORF1a, and ORF1b) available for a larger number of strains (i.e., Canadian strains). In all the analyses the most likely scenario is that the introduction of the virus to Uruguay occurred from Europe (**Supplementary Figures S1A–D**). In addition, the estimated date for this introduction, obtained with the ORF1ab and partial polymerase genomic region (ORF1b) (**Supplementary Figures S1A,D**), was similar to that obtained with the complete coding region, whereas the estimated date of introduction obtained with ORF2 and ORF1a was earlier but with wider 95% HPD interval (**Supplementary Figures S1B,C**). An introduction of the sub-lineage NeuroS1 directly to Canada from Europe, with subsequent spread to United States and Japan, is also plausible, s shown in **Supplementary Figure S1D**.

Further investigations are needed to assess the geographic distribution, pathogenic mechanisms (particularly mechanisms of transmission and entry), molecular epidemiology, and potential interspecies transmission of neuroinvasive astroviruses.

DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, MK386569.

AUTHOR CONTRIBUTIONS

FG, RDC, and MC contributed with the conception of the study. FG and RDC performed the pathological examination and sampling. PP performed the *in situ* hybridization. FU performed the immunohistochemistry. LM, RC, and MC performed molecular virology testing. MC performed the sequence and phylogeographic analyses and associated figures. FG and PP obtained the histologic images. MF performed the bacterial cultures. FG and MC wrote the first draft of the manuscript. RDC, PP, FU, LM, MF, and RC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01240/full#supplementary-material

FIGURE S1 | Maximum clade credibility trees (MCCTs) obtained by analysis of the full-length ORF1ab **(A)**, full-length ORF2 **(B)**, full-length ORF1a **(C)**, and partial ORF1b **(D)**. The color of the branches represents the most likely country where the ancestors circulated, posterior probability values are shown in the branches, and the numbers in each node represent the years of origin for each clade with the 95% HPD interval. Sub-lineages are indicated with labels.

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The reviewer TS declared a past co-authorship with one of the authors PP to the handling editor.

B.5. Estudio de norovirus en muestras clínicas

Artículo 7. Detección, factores de riesgo y diversidad molecular de norovirus GIII en terneros en Uruguay

Este artículo tuvo como objetivo caracterizar a norovirus bovino en Uruguay, determinando la frecuencia de detección, los factores de riesgo asociados a la infección, así como también la diversidad genética de este virus.

La frecuencia de detección fue de 66% (503/761); se detectó mayormente en terneros lecheros que terneros de carne ($p<0,01$). Se detectó tanto en terneros con diarrea como en terneros sin diarrea. Los terneros menores a 2 semanas de edad fueron infectados más frecuentemente que terneros mayores ($p<0,01$). La carga viral detectada en las muestras positivas fue muy variable, con valores de C_t entre 7,84 y 40,0. Particularmente, 35 muestras tuvieron una carga viral muy alta ($C_t \leq 15$), mientras que 8 de ellas mostraron un $C_t < 10$.

En cuanto a la variabilidad genética, detectamos la presencia de los dos genotipos ya establecidos, GIII.1 y GIII.2, así como también tres cepas recombinantes (GIII.P1/GIII.2), y una cepa con una nuevo genotipo recientemente descrito.

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Research paper

Detection, risk factors and molecular diversity of norovirus GIII in cattle in Uruguay

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ABSTRACT

Uruguay is a leading exporter of bovine meat and dairy products, and cattle production is one of the principal economic backbones in this country. A main clinical problem faced by livestock farmers is neonatal calf diarrhea (NCD); however, causes of NCD have not been extensively studied in Uruguay. Bovine norovirus (BoNoV) has been proposed as one of the possible etiologies of NCD as experimentally infected calves developed diarrhea and enteropathy, although limited information is available from field surveys. The aims of this study were to determine the frequency of infection, to investigate possible risk factors, and to determine the molecular diversity of BoNoV in Uruguay. A total of 761 samples of feces or intestinal contents from dairy and beef calves were analyzed through RT-qPCR. The overall frequency of detection of BoNoV was 66.1% with higher frequency in dairy (70.5%) than beef (15.9%) calves ($p < 0.01$). BoNoV was detected similarly in diarrheic (78.8%) and non-diarrheic (76.2%) dairy calves ($p = 0.50$). Calves ≤2 weeks of age (84%) were infected more often than older (62.7%) calves ($p < 0.01$). Phylogenetic analysis confirmed the presence of GIII.1 and GIII.2 genotypes. In addition, we reported the circulation of recombinant strains and the detection of a strain with the recently described novel VP1 genotype. This study represents the first report describing the circulation, the associated risk factors, and the molecular diversity of BoNoV in Uruguay.

1. Introduction

A main problem that the livestock industry must face is neonatal calf diarrhea (NCD), a complex and multifactorial clinical syndrome of worldwide distribution. Although NCD affects both beef and dairy cattle, it is particularly important in dairy farming as it represents the major cause of mortality of calves before weaning (Urie et al., 2018). NCD leads to economic losses to the livestock industry due to a negative impact on animal wellness with short- and long-term effects on production (Waltner-Toews et al., 1986; Donovan et al., 1998). The causes of NCD outbreaks are poorly known and rarely investigated; also little is known about the prevalence, relative importance, possible interrelationships and pathogenic effects of the numerous microorganisms that have been shown or suggested as a cause of diarrhea (Selman, 1981).

Bovine noroviruses (BoNoVs) have not captured the attention that other pathogens have received and are not included in routine diagnosis for NCD, so their impact on livestock health and production remains unclear (Di Felice et al., 2016). BoNoVs were discovered in 1978, and their pathogenicity is mainly due to lesions in the small intestine,

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including villus atrophy with loss and attenuation of the villus epithelium, inducing diarrhea (Woode and Bridger, 1978; Jor et al., 2010; Otto et al., 2011; Jung et al., 2014).

Classified within the genogroup III (GIII) of the *Norovirus* genus in the *Caliciviridae* family (Scipioni et al., 2008), BoNoV are non-enveloped viruses with a single stranded RNA genome of positive polarity of approximately 7.5 kb containing 3 ORFs. Transmission is mainly sustained by the fecal-oral route, and low infectious doses as well as the great diversity of strains increase the risk of infection (Scipioni et al., 2008).

Although BoNoV is studied to a much lesser extent than other viruses that are well known causative agents of NCD such as rotavirus and coronavirus, several studies confirm that BoNoVs are widely present in cattle and sometimes at a high frequency in cases of diarrhea in different countries (van Der Poel et al., 2000; Deng et al., 2003; van Der Poel et al., 2003; Milnes et al., 2007); they are also detected in non-diarrheic calves (Jor et al., 2010). In addition, a serological study indicated that exposure to BoNoV can reach over 99% of the analyzed samples (Deng et al., 2003).

There have been recognized three genotypes within GIII, namely GIII.1, GIII.2, and GIII.3, being GIII.1 and GIII.2 associated to bovine norovirus, and GIII.3 to ovine norovirus. In addition, several studies have demonstrated the circulation of recombinant strains, with the recombination breakpoint in the ORF1-ORF2 junction genomic region (Bull et al., 2007). Both genotypes GIII.1 and GIII.2, formerly referred to as Jena virus and Newbury-2 virus, respectively, have been shown to be diarrheagenic when inoculated experimentally into calves (Woode and Bridger, 1978; Jor et al., 2010; Otto et al., 2011; Jung et al., 2014).

Uruguay is one of the main exporters of bovine meat (FAO, 2018) and dairy products (IDF, 2013), and cattle production is one of the main economic backbones in this country (DIEA, 2019), however, there are no studies on BoNoV in cattle. The aims of this study were to determine the frequency of infection, to investigate possible risk factors, and to determine the molecular diversity of BoNoV in calves in Uruguay.

2. Materials and methods

2.1. Samples and fecal suspensions

A total of 761 samples of feces (699) or intestinal contents (62) were collected from dairy (717) and beef (44) calves in Uruguay. The intestinal contents were collected from diarrheic calves that died naturally, and all 699 fecal samples were from live calves. Risk factors for BoNoV infection were only evaluated in dairy calves, as insufficient data was available from beef calves. Fecal samples from dairy calves were categorized as diarrheic (208) and non-diarrheic (235) at the time of sampling (this information was missing for 443 samples); 209 samples came from calves in dairy herds that reported vaccinating the dams against NCD, while 203 came from calves in dairy herds where vaccination against NCD was not practiced (for 412 samples this information was unavailable). Regarding the age of the dairy calves, 127 were up to 1 week old, 180 were in the second week of life, 96 were in the third week of life, 28 were in the fourth week of life, and 10 were more than 4 weeks old. Samples were collected in 2015 ($n = 39$), 2016 ($n = 490$), 2017 ($n = 185$), and 2018 ($n = 47$).

2.2. Viral RNA extraction and reverse transcription

From all samples, suspensions were obtained after diluting 1:10 (v:v) in phosphate-buffered saline solution, and supernatants were collected after centrifugation at 3000*g* for 20 min at 4 °C. Viral RNA was extracted using QIAamp® cador® Pathogen Mini Kit (Qiagen®), following the manufacturer's instructions. Reverse transcription (RT) was carried out with RevertAid® Reverse Transcriptase (Thermo Fischer Scientific®) and random hexamers primers (Qiagen®), following manufacturer's instructions. All RNAs and cDNAs were stored at −80 °C until further viral analyses.

2.3. BoNoV screening and sequencing

Screening of the samples for GIII BoNoV identification was carried out through a real time polymerase chain reaction (qPCR) targeted to the junction between the ORF1 and ORF2, which is a highly conserved genomic region. Primers, probe and real time PCR conditions were used as described elsewhere (Wolf et al., 2007).

In order to determine the genotypes circulating in the Uruguayan calves, 50 qPCR-positive samples were selected randomly and subjected to amplification of a 517-bp fragment (Wolf et al., 2007). Briefly, 12.5 μL of MangoMix™ (Bioline®), 5 μL of cDNA, 4.5 μL of nuclease-free water, 1 μL of dimethyl sulfoxide, 1.0 μL of 10 μM SW GIII forw primer and 1.0 μL of 10 μM NVGIIIrseq primer were mixed in 0.2 mL PCR tubes. PCR products were visualized in 2% agarose gels and positive samples were purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen®) according to the manufacturer's instructions; both DNA strands were sequenced at Macrogen Inc. (Seoul, South Korea). Sequences were deposited in GenBank with accession numbers: MT227833-MT227846, and MT765190-MT765209.

2.4. Phylogenetic analysis

A phylogenetic analysis was performed in order to determine the BoNoV genotypes circulating in Uruguay. Database sequences were downloaded using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequences alignment was obtained with ClustalW in MEGA 7 software (Kumar et al., 2016). The nucleotide substitution model that best fit our data and the maximum likelihood tree was obtained with IQ-TREE (Trifinopoulos et al., 2016).

2.5. Recombination analysis

The SimPlot program was used to determine the presence of evidence of recombination in the sequences, with a window size of 50 and a step size of 25. Jena and Newbury2 strains were used as reference sequences for GIII.1 and GIII.2 genotypes, respectively. In order to confirm the evidence, a nucleotide identity matrix and phylogenetic analysis were performed with partial sequences before and after the recombination breakpoint suggested by SimPlot analysis.

2.6. Statistical analyses

Categorical data was evaluated with jamovi software (available at https://www.jamovi.org/) using 2 × 2 contingency tables and through Pearson's Chi-squared test; in multiple comparisons, the Bonferroni correction was applied. Relative risk (RR) and 95% confident intervals (CI) were calculated with jamovi software. Numerical data was evaluated with jamovi software using the Shapiro-Wilk test for normality (Shapiro and Wilk, 1965) and when the null hypothesis was rejected Mann-Whitney *U* test was used (Mann and Whitney, 1947). In all tests, differences were considered statistically significant if the obtained *p*-value was ≤ 0.05. Graphics were generated using Microsoft® Office Excel, and the line of tendency that best fit the data (evaluated by the R^2 value) was obtained with the same program.

3. Results

3.1. BoNoV detection and risk factors

Bovine norovirus (GIII) was detected in 66.1% (503/761) of the analyzed samples. The frequency of detection was significantly higher in dairy 70.5% (462/655) than beef (15.9%, 7/44) calves (RR: 2.85, 95% CI: 2.4–3.4; $p < 0.00001$) (Fig. 1a). BoNoV was detected in 67.1% (469/699) and 54.8% (34/62) of the samples of feces (live calves) and

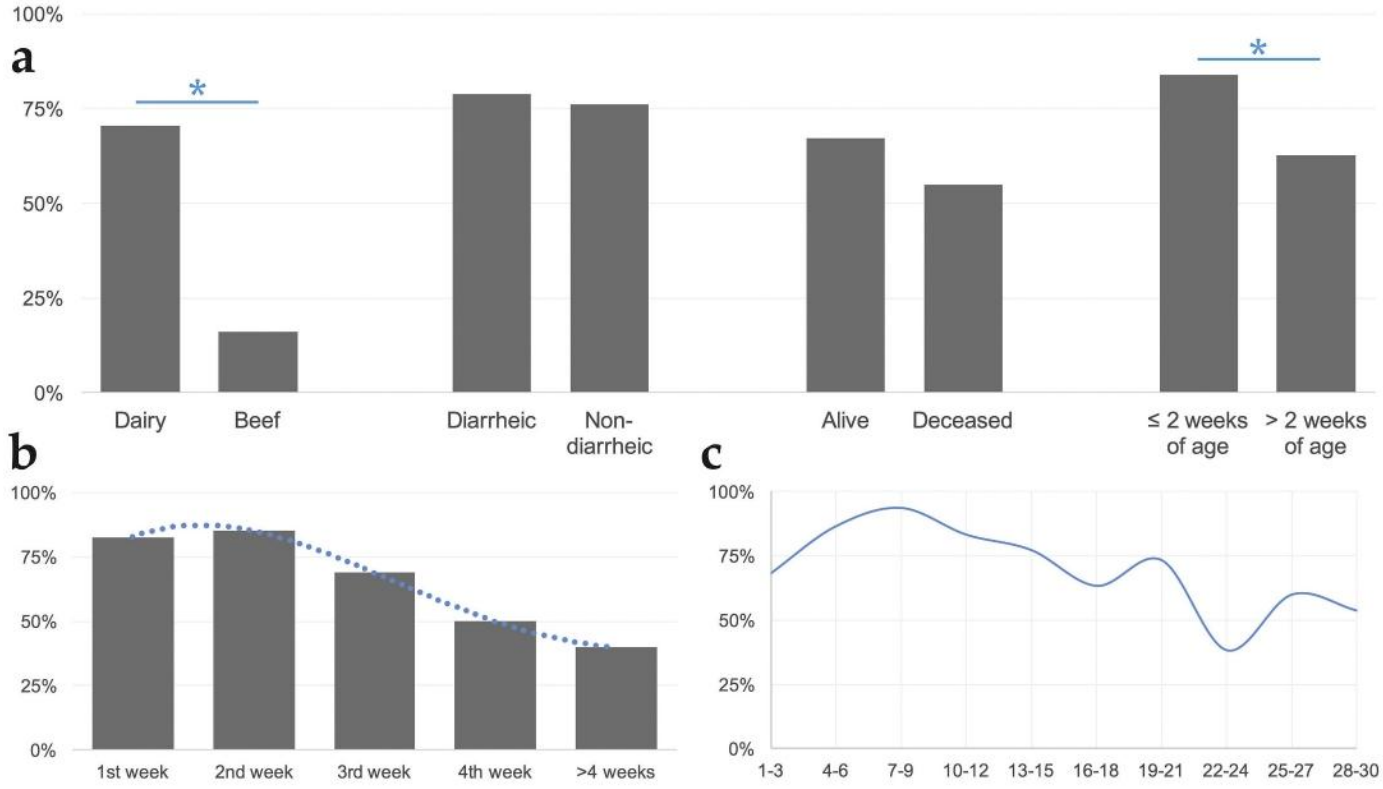

Fig. 1. Bovine norovirus (BoNoV) detection. a) Comparison of frequency of BoNoV detection in dairy vs. beef calves, diarrheic vs. non-diarrheic, live vs. deceased calves, and calves ≤2 vs. >2 weeks of age. Comparisons with statistical significant differences are indicated by an asterisk ($p < 0.00001$). b) Frequency of BoNoV detection according to the calves age in weeks; a line of tendency (polynomial regression) was adjusted with an $R^2 = 1.00$. c) Frequency of BoNoV detection according to the calves age in days.

intestinal contents (deceased calves), respectively, this difference being non-significant ($p = 0.051$) (Fig. 1a).

The proportion of BoNoV-positive samples was higher in diarrheic 78.8% (164/208) than non-diarrheic 76.2% (179/235) calves, although this difference was not statistically significant ($p = 0.50$) (Fig. 1a). Calves born to dams vaccinated against NCD (vaccines include several pathogens but not BoNoV) showed a frequency of detection of 82.8% (173/209) while calves born to dams unvaccinated showed a frequency of detection of 77.3% (157/203), this difference was not statistically significant ($p = 0.17$).

Calves ≤2 weeks of age were more often infected by BoNoV (84%, 258/307) than older calves (62.7%, 84/134), and this difference was statistically significant (RR: 2.06, 95%CI: 1.57–2.69; $p < 0.00001$) (Fig. 1a). The frequency of BoNoV detection in calves in their first, second, third, fourth and after the fourth week of life was 82.7% (105/127), 85% (153/180), 68.8% (66/96), 50% (14/28), 40% (4/10), respectively (Fig. 1b). The frequency of BoNoV was significantly higher between the age groups: 1vs4, 1vs > 4, 2vs3, 2vs4, and 2vs > 4, so BoNoV was more frequently detected in calves in their first two weeks of age. This frequency declined with the age, as observed in a line of tendency obtained by a polynomial regression ($R^2 = 1.00$) (Fig. 1b). Similarly, when the frequency of BoNoV detection was analyzed by the age of the calves in days, a peak between days 7 and 9 was observed (Fig. 1c).

The mean age of the positive calves to BoNoV (11.0 days) was significantly lower than the mean age of the negative ones (14.4 days; $p < 0.001$) (Fig. 2a); in addition, in the diarrheic calves, the mean age of the positive calves to BoNoV (10.3 days) was significantly lower than the

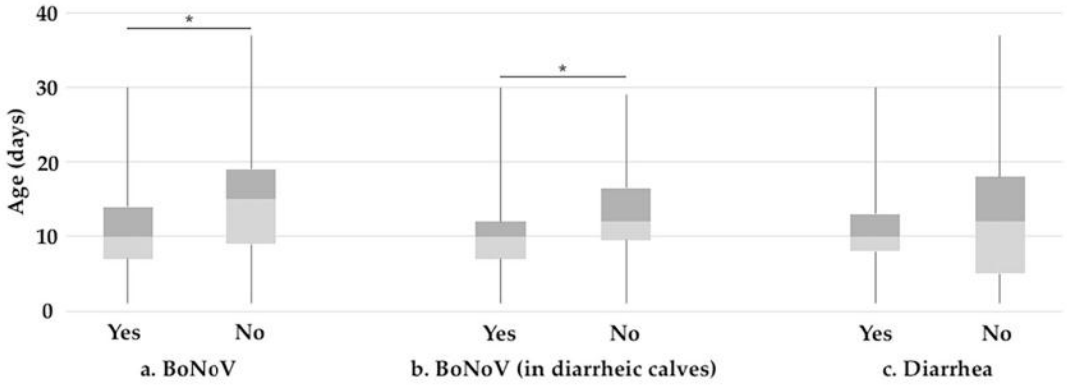

Fig. 2. Box and whiskers plots of age (in days) with different outcomes are shown. a) Age in days of BoNoV-positive and BoNoV-negative calves. b) Age in days of BoNoV-positive and BoNoV-negative diarrheic calves. c) Age in days of diarrheic and non-diarrheic calves. *Indicate statistically significant differences ($p < 0.05$).

mean age of the negative ones (13.1 days; $p < 0.001$) (Fig. 2b). Diarrheic calves (mean age of 10.9 days) were younger than non-diarrheic (mean age of 12.5 days) (Fig. 2c), and diarrheic BoNoV-positive calves were younger (mean age of 10.3 days) than non-diarrheic BoNoV-positive calves (mean age of 11.6 days), but these differences were not statistically significant ($p = 0.074$ and $p = 0.093$, respectively).

The range of the Ct-values was 7.84–40.0 (Fig. 3) with a mean Ct-value of 27.8 (SD 7.4) and a median Ct-value of 29.3. Thirty-five samples showed high viral load with Ct-values <15, 237 showed Ct-values between 15 and 30, and 246 showed low viral load with Ct-values >30 (Fig. 3). In addition, we compare the Ct values among calf groups; we did not observe differences in the Ct values between diarrheic and non-diarrheic calves, and between alive and deceased calves. On the other hand, we observed lower Ct values in dairy (mean ± SD, 27.7 ± 7.4) than beef (32.2 ± 7.9) calves ($p = 0.026$), and lower Ct values in calves ≤2 weeks of age (26.1 ± 8.0) than older calves (30.2 ± 6.1, $p < 0.001$).

3.2. Genetic diversity of BoNoV in Uruguay

Thirty-four sequences were obtained from the 50 subjected to conventional PCR amplification. Twelve strains could not be sequenced because no or very low amplification, and four sequences were excluded from the analysis because showed chromatograms with double peaks, maybe due to coinfection. GIII.1 and GIII.2 genotypes were identified in four and 23 samples, respectively (Fig. 4). Three strains clustered within GIII.2, but together with recombinant strains, and one strain clustered together with Chinese strains with evidence of recombination and possessing a novel VP1 genotype (Fig. 4). Three sequences were shorter so were excluded from the analysis, but the three were GIII.2 (data not shown).

As some Uruguayan strains clustered together with recombinant strains, we performed several analyses to determine if these were also recombinant. SimPlot analysis showed evidence of recombination in the strains Bo/LVMS3019/2016/UY, Bo/LVMS3752/2017/UY, Bo/LVMS3970/2017/UY, and Bo/LVMS3974/2017/UY (Fig. S1). The recombination breakpoints were observed in the junction ORF1-ORF2 in all the four strains. We confirmed these evidence with nucleotide sequences identity matrices: one performed with the partial 3′ end of the polymerase (RdRp, upstream the recombination breakpoints, Table 1), and another with the partial 5′ end of the capsid (downstream the recombination breakpoints, Table 1). This was confirmed also by phylogenetic analyses performed with the same two genomic regions used for the identity matrices (Fig. S2).

4. Discussion

The diarrheagenic and enteropathogenic potential of BoNoV have been well established in experimental infections in calves (Woode and Bridger, 1978; Jor et al., 2010; Otto et al., 2011; Jung et al., 2014), but under natural conditions has not yet been thoroughly studied, being relegated behind other pathogens such as rotavirus A, coronavirus, enterotoxigenic and enteropathogenic *Escherichia coli*, *Cryptosporidium parvum*, and *Salmonella enterica*, among others. However, studies have demonstrated its endemicity in several countries (Jor et al., 2010; Deng et al., 2003; van der Poel et al., 2003; Oliver et al., 2007; Mauroy et al., 2009; Thomas et al., 2014) and its role as enteric pathogen in calves (Di Felice et al., 2016), endorsing the need for epidemiological surveillance of BoNoV.

The frequency of BoNoV detection of 66.1% in this study was higher than the reported for other enteric viruses such as bovine rotavirus A (57%) (Castells et al., 2020), bovine coronavirus (7.8%) (Castells et al., 2019a) and bovine astrovirus (26%) (Castells et al., 2019b) in cattle in Uruguay, in accordance with studies that have demonstrated that BoNoV could be the most commonly detected pathogen in calves' feces (Cho et al., 2013). In addition, the frequency observed in our study is higher than the reported for BoNoV in other countries including Argentina where the frequency of detection was 3.3% (Ferragut et al., 2016), indicating that BoNoV is probably a belittled pathogen involved in calf diarrhea in Uruguay. The detection method used in our work (qPCR), probably has a higher sensitivity and specificity than the conventional PCR used in Argentina (Ferragut et al., 2016), which could partially explain this difference.

Despite their diarrheagenic and pathogenic potential under experimental conditions, whether BoNoVs represent a significant or even rare cause of spontaneous disease in cattle has not been widely studied. In our study the frequency of BoNoV detection was similar in diarrheic and non-diarrheic calves, as observed in The Netherlands (van der Poel et al., 2003); in USA, a higher frequency was observed in diarrheic calves, suggesting that this virus may be a significant contributor to calf diarrhea (Cho et al., 2013). Further investigations are needed to elucidate the clinical significance of the different BoNoV genotypes in spontaneous outbreaks of disease and eventual impact to the livestock industry. Several studies have demonstrated the prolonged shedding of the virus, before and after the manifestation of the diarrhea (Jor et al., 2010; Jung et al., 2014). Asymptomatic calves can spread the virus and their identification in the field is extremely difficult, which could have influenced the high frequency of detection observed in our study. The management of the calves in intensive systems is even more complicated, because separating only the diarrheic calves may not prevent the virus dispersion at the herd.

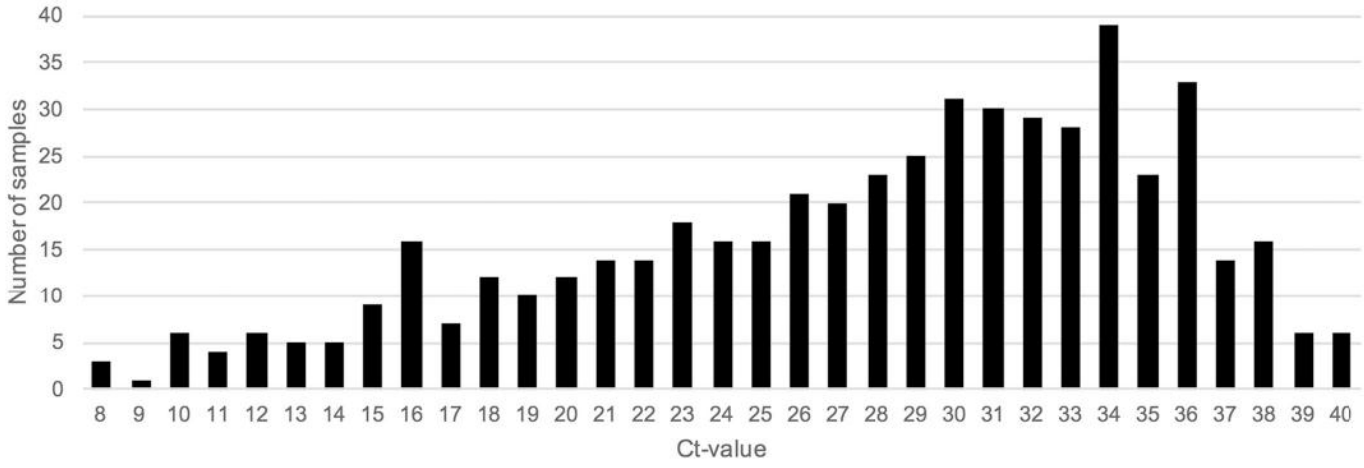

Fig. 3. Distribution of Ct-values (rounded to nearest integer value) of the BoNoV-positive samples.

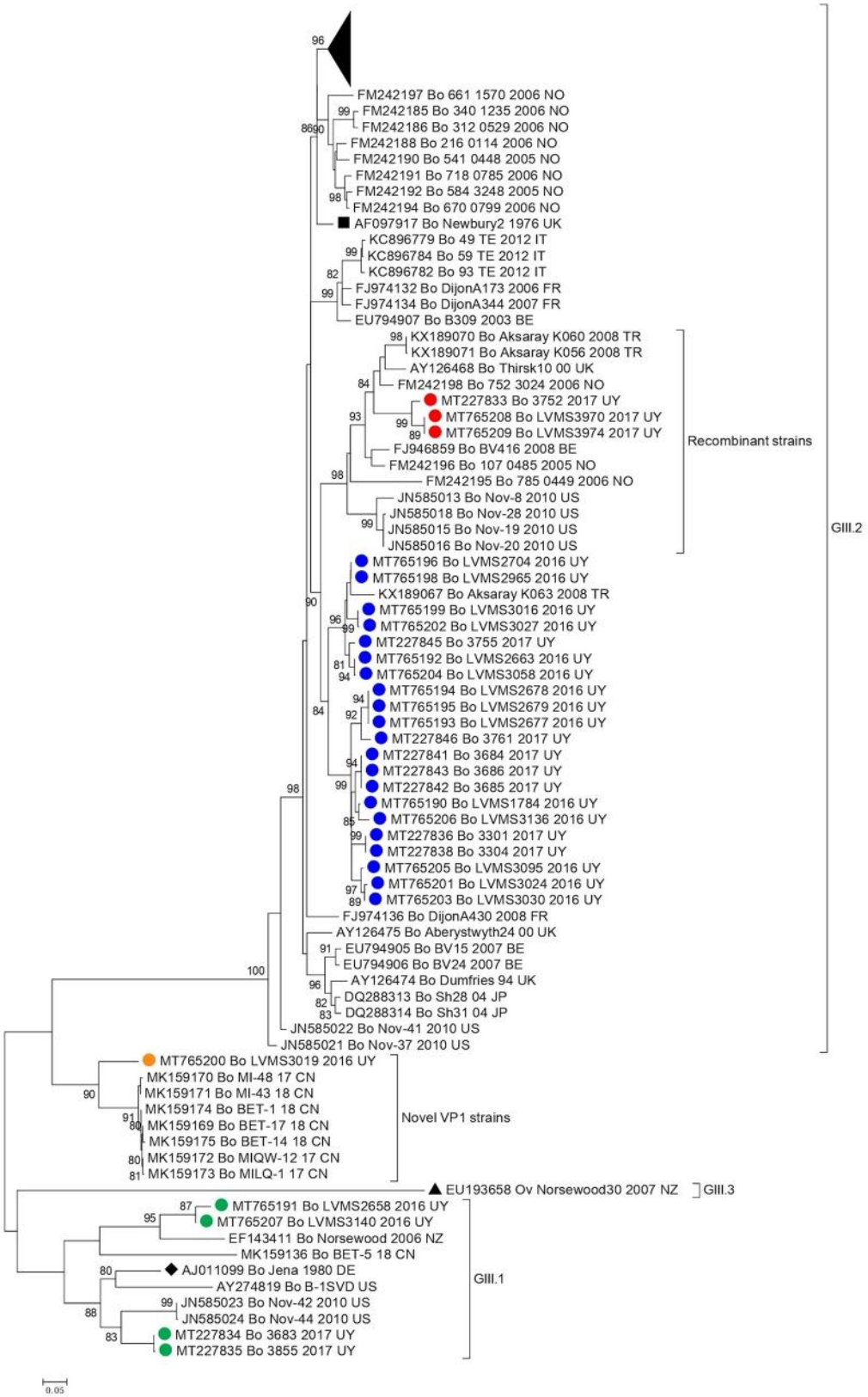

Fig. 4. Maximum likelihood tree with the TNe + I + G4 model obtained with IQ-TREE. Reference strains: Jena (GIII.1, black rhombus), Newbury2 (GIII.2, black square), and Norsewood30 (GIII.3, black triangle). Uruguayan strains are indicated with green circle (GIII.1), blue circle (GIII.2), orange circle (novel VP1), and red circle (recombinants GIII·P1-GIII.2). aLRT values higher than 80 are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sequence identity of Uruguayan recombinant strains compared with reference strains.

	3′ end of RdRp			5′ end Capsid		
	Jena (GIII.1)	Newbury2 (GIII.2)	BET-17 (Novel VP1)	Jena (GIII.1)	Newbury2 (GIII.2)	BET-17 (Novel VP1)
MT765208_Bo/LVMS3970/2017/UY	**0.986**	0.934	0.947	0.740	**0.857**	0.784
MT765209_Bo/LVMS3974/2017/UY	**0.986**	0.934	0.947	0.740	**0.857**	0.784
MT227833_Bo/3752/2017/UY	**0.986**	0.934	0.947	0.745	**0.864**	0.774
MT765200_Bo/LVMS3019/2016/UY	0.947	**0.973**	**0.986**	0.808	0.782	**0.876**

The obtained Ct-values ranged from 7.84 to 40.0, similar to the reported in Norway, where Ct-values ranged from 6.9 to 39.7 (Jor et al., 2010). Interestingly, we observed a right skewed distribution of Ct-values, as previously reported, which may reflect prolonged low viral shedding post-clinical illness (Jor et al., 2010). Calves can be reinfected, prolonging the viral shedding without clinical signs, acting as reservoirs (Jor et al., 2010), which could reflect the high frequency of detection in non-diarrheic calves.

We observed that dairy calves were more often infected by BoNoV and with higher viral loads than beef calves, so studies about possible management conditions, geographic influence, and/or calves' genetic factors influencing this observation are encouraged.

Another factor studied was vaccination against NCD; it is important to clarify that vaccines do not include BoNoV, as there are no commercial vaccines available against this virus. BoNoV detection was higher in calves born to vaccinated dams but the difference was not statistically significant. Studies in humans (where vaccines against rotavirus A –RVA- but not norovirus are available as occurs in cattle) have demonstrated that vaccination against RVA have lead a reduction in RVA detection, leaving norovirus as the main cause of gastroenteritis in children (Hemming et al., 2013; Payne et al., 2013; Hemming-Harlo et al., 2016). In contrast, a study in diarrheic calves in France showed that vaccination against RVA did not promote the emergence of BoNoV (Kaplon et al., 2013), which is in concordance with the observed in our study. On the other hand, vaccines seem not to be effective against RVA in cattle (Castells et al., 2020), which could have influenced that no difference in BoNoV detection was observed.

The calves' age was analyzed as a factor that could influence the detection of BoNoV, and we observed that BoNoV was most commonly detected in the first two weeks of age, as observed in the USA (Smiley et al., 2003; Cho et al., 2013), indicating that younger calves are more frequently infected by BoNoV, and in this sense, the mean age of calves positive to BoNoV was lower than that of calves negative to the virus. In addition, the viral load was higher in calves ≤2 weeks of age than older. Taken together, this data suggests that younger calves are more susceptible to BoNoV infection.

The phylogenetic analysis allowed to confirm that both main genotypes of BoNoV, GIII.1 and GIII.2, were circulating in Uruguay. The predominant genotype was GIII.2, as commonly observed in other countries (Mauroy et al., 2009; Kaplon et al., 2013; Thomas et al., 2014). Unfortunately, the sequence of the tentatively new genotype described in Argentina (Ferragut et al., 2016) was not available, thus, we could not determine if this genotype was circulating in Uruguay. The Uruguayan strains clustered in two lineages within GIII.1, in a divergent lineage divided in two sub-lineages within GIII.2, in a lineage together with GIII·P1/GIII.2 strains, and in a lineage together with Chinese strains recently described with a novel VP1 genotype, denoting a high genetic heterogeneity. The region amplified allowed the detection of possible recombinant strains, that were then confirmed by other specific methods. This region, then, is suitable for the detection and analysis of the genetic diversity of BoNoV (Jor et al., 2010). However, for a more accurate classification, complete genomes should be obtained.

Four Uruguayan strains showed recombination evidence, confirming the wide circulation of recombinant strains worldwide (Han et al., 2004; Oliver et al., 2004; Bull et al., 2007; Mauroy et al., 2009; Jor et al., 2010; Di Martino et al., 2014; Ferragut et al., 2016; Mohamed et al., 2018; Karayel-Hacioglu and Alkan, 2019; Wang et al., 2019). This widely distribution of highly similar recombinant strains, may be due to ancestral recombination events that later spread widely in cattle. As for HIV (Reis et al., 2019), widely dispersed recombinant strains should be named BoNoV circulating recombinant forms (BoNoV CRFs), in order to facilitate the classification. A limitation of this study was the short length of the partial 3′ end of the polymerase, so the results related to recombination should be taken with caution. Three of the four recombinant strains were GIII.1/GIII.2, the most widely dispersed CRF. Most of the GIII.1/GIII.2 recombinant clustered together, further supporting a common origin. Interestingly, one strain clustered with recombinant strains with a novel VP1 genotype (Wang et al., 2019), which suggests that probably this novel VP1 genotype is widely dispersed, and maybe has been overlooked.

In conclusion, this was the first study on BoNoV conducted in Uruguay, and revealed a high frequency of BoNoV infection in diarrheic and non-diarrheic calves, with higher frequency in dairy than in beef calves. Calves ≤2 weeks of age were infected more often than older calves. Both main genotypes, GIII.1 and GIII.2, were identified, and four recombinant strains were described. Despite no clear association was found with NCD, the results of our study indicate that BoNoV may be a belittled pathogen involved in calf diarrhea.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104613.

Declaration of Competing Interest

None.

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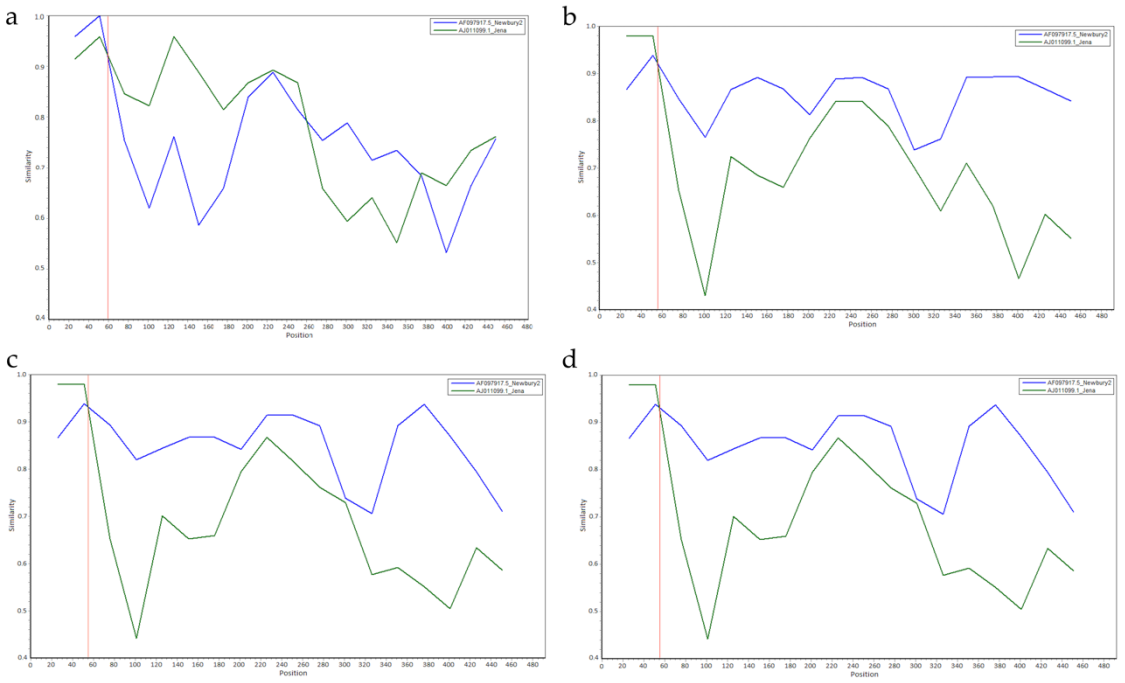

Figure S1. The nucleotide similarity plots for a) Bo/LVMS3019/2016/UY, b) Bo/LVMS3752/2017/UY, c) Bo/LVMS3970/2017/UY, and d) Bo/LVMS3974/2017/UY are shown. Jena and Newbury2 were used as reference strains for the confirmed genotypes GIII.1 and GIII.2, respectively. The recombination breakpoint is shown with a red line.

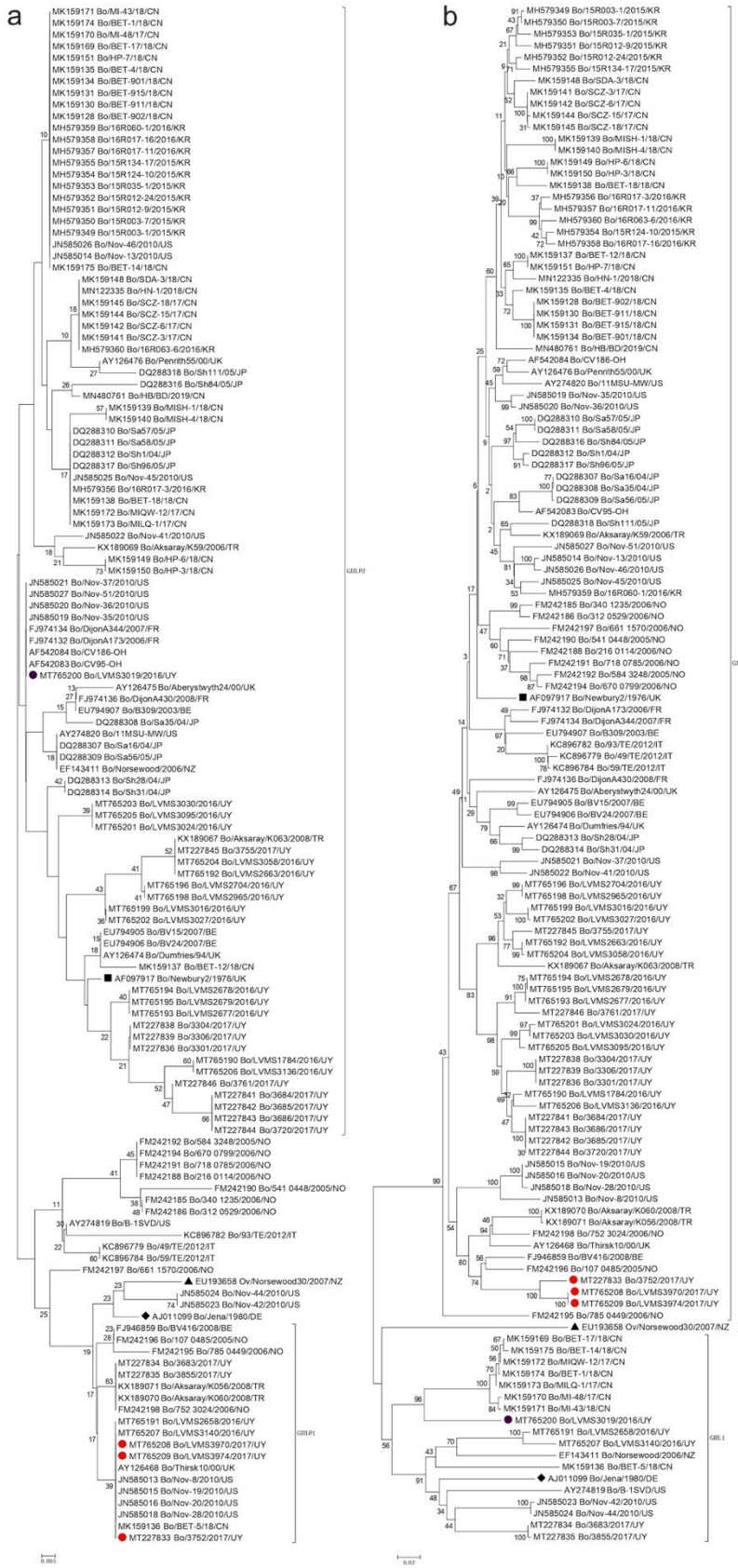

Figure S2. Neighbor joining trees with the K2+G model and 1000 bootstrap replicas were obtained with MEGA7. a) 3'end of RdRp and b) 5'end of VP1. Uruguayan recombinant strains are indicated with red and purple circles. Reference strains of GIII.1, GIII.2 and GIII.3 genotypes are indicated with black square, rhombus, and triangle, respectively.

Artículo 8. Historia evolutiva y dinámica espacio-temporal de norovirus GIII: desde la emergencia hasta la clasificación en 4 genotipos

Este artículo tuvo como objetivo clasificar a los norovirus pertenecientes al genogrupo III (GIII). La clasificación de este virus por parte del grupo de trabajo para la clasificación de norovirus (*Norovirus Classification Working Group*, NCWG), se ha basado fundamentalmente en los norovirus que afectan a los humanos, quedando relegada la correcta y completa clasificación de GIII.

Como resultado principal, se determinó la existencia de 4 genotipos distintos de GIII, sumándose un nuevo genotipo, aún no incluido por el NCWG en su clasificación. Además, se reconstruyó la historia evolutiva y la dinámica espacio-temporal, determinando el origen temporal del genogrupo y el continuo surgimiento de nuevos genotipos a lo largo del tiempo. Se determinó que los dos genotipos principales hasta el momento, se encuentran probablemente en una fase de disminución poblacional, mientras que el nuevo genotipo GIII.4, se encuentra emergiendo y con esto aumentando su número poblacional.

De manera interesante, nuestro país es probablemente la región geográfica donde tuvo origen este genogrupo, así como también 2 de los 4 genotipos descritos hasta el momento. Por otro lado, se describió el proceso por el cual una cepa recombinante se dispersó mundialmente.

Estos resultados fueron publicados en la revista Transboundary and Emerging Diseases: Castells M, Cristina J, Colina R. Evolutionary history and spatiotemporal dynamic of GIII norovirus: from emergence to classification in 4 genotypes. Transbound Emerg Dis. 2021 1-8. doi: 10.1111/tbed.14168.

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ORIGINAL ARTICLE

Evolutionary history and spatiotemporal dynamic of GIII norovirus: From emergence to classification in four genotypes

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Comisión Sectorial de Investigación Científica

Abstract

Noroviruses belong to a genetically diverse group of viruses infecting a wide range of mammalian host species, and those detected in cattle and sheep are classified within genogroup III (GIII). The current classification of norovirus in genogroups and genotypes is based on phylogenetic clustering and average distances within and between these phylogenetic clusters; however, the classification studies have been focused mainly on human norovirus, being GIII norovirus relegated. Due to the increasing number of studies on GIII norovirus, the need of an updated and extensive classification is evident. The aim of this study was to update the classification of norovirus within GIII, to describe the emergence of a circulating recombinant strain, and to reconstruct the evolutionary history of this genogroup. Two P-types (GIII.P1–2) and four genotypes (GIII.1–4) were described. For the genogroup GIII, the evolutionary rate estimated was 2.78E-3 s/s/y (95%HPD, 1.79E-3 s/s/y–3.78E-3 s/s/y), and the tMRCA was estimated around 1500 (95%HPD, 1247–1688). Despite the long history of this genogroup, the genotypes detected at present emerged in the last 100 years. Interestingly, most of the recombinant GIII.2P[1] strains detected worldwide were originated from a single recombination event and this recombinant strain was later dispersed through the world. Finally, our results indicate that a scenario of genotypes replacement through the time is highly probable.

KEYWORDS
classification, emergence, evolutionary history, genotypes, GIII, norovirus

1 | INTRODUCTION

Noroviruses belong to a genetically diverse group of viruses infecting a wide range of mammalian host species (Chhabra et al., 2019). This genetic diversity found in noroviruses requires a correct and updated classification. Recently, an update in the classification of norovirus genogroups and genotypes was published (Chhabra et al., 2019). Within genogroup III (G.III), there have been defined three genotypes, named GIII.1, GIII.2, and GIII.3 (Wang et al., 2019). However, there are still some gaps in the classification of GIII of norovirus.

Noroviruses detected in cattle are classified within genotypes GIII.1 and GIII.2, while noroviruses detected in sheep are classified within genotype GIII.3. The reference strains for these genotypes are Bo/Jena/1980/DE, Bo/Newbury2/1976/UK, and Ov/Norsewood30/2007/NZ, respectively. Recently, a novel VP1 genotype has been described in China (Wang et al., 2019) and Uruguay (Castells et al., 2020). In addition, several recombinant strains have been detected, with a worldwide distribution (Castells et al., 2020).

Taxonomically, the *Norovirus* genus belong to the *Caliciviridae* family (Scipioni et al., 2008). Noroviruses are nonenveloped with a single-stranded RNA genome of positive polarity of approximately 7.5 kb containing three ORFs. Transmission is mainly sustained by the fecal-oral route, and low infectious doses as well as the great diversity of strains increase the risk of infection (Scipioni et al., 2008).

Noroviruses are widely present in cattle and sometimes at a high frequency in cases of diarrhea in different countries (Castells et al., 2020; Deng et al., 2003; Milnes et al., 2007; van Der Poel et al., 2000; van Der Poel et al., 2003). In addition, a serological study indicated that exposure to BoNoV can reach over 99% of the analyzed samples (Deng et al., 2003). Furthermore, gnotobiotic calves inoculated with both GIII.1 and GIII.2 noroviruses developed diarrhea (Jor et al., 2010; Jung et al., 2014; Otto et al., 2011; Woode & Bridger, 1978), thus confirming their potential as contributors in the neonatal calf diarrhea syndrome, a main clinical problem faced by livestock farmers worldwide. GIII norovirus causes persisting mild diarrhea and prolonged fecal virus shedding, which might partially explain the endemicity of this virus in cattle (Jung et al., 2014).

Although noroviruses are generally detected in a species-specific manner (depending on the genogroup), calves inoculated with GII (isolated from human) not only were infected but also developed diarrhea and showed intestinal lesions (Souza et al., 2008), raising a question about the possibility of zoonosis.

The current classification of norovirus in genogroups and genotypes is based on phylogenetic clustering and the average distances within and between these phylogenetic clusters. In this sense, the average distance between all the sequences within a newly identified cluster and its nearest established cluster should not overlap within 2 standard deviations of each other (2xSD criterion) (Chhabra et al., 2019; Kroneman et al., 2013). However, the classification studies have been focused mainly on human norovirus, being GIII norovirus relegated.

Due to the increasing number of studies on GIII norovirus, the need of an updated and extensive classification is evident. The aim of this study was to update the classification of norovirus within GIII in genotypes, to determine circulating recombinant strains, and to reconstruct the evolutionary history of this genogroup.

2 | METHODOLOGY

2.1 | Sequences used for analyses

All the available GIII complete genomes, complete capsid, complete RdRp, and partial capsid sequences were downloaded from GenBank (last access: November 2020). Partial capsid sequences correspond to the first 383 nucleotides of the ORF2. Datasets were constructed for the mentioned genomic regions, with both nucleotide and amino acid sequences. Multiple sequences alignments were obtained using ClustalW, available in MEGA 7.0 software (Kumar et al., 2016). RDP4 program (Martin et al., 2015) was used to discard recombinant sequences (in the analyzed region).

2.2 | Phylogenetic analyses

Maximum likelihood (ML) trees were obtained by using the previously obtained alignments. Briefly, the best substitution model and the ML tree for each dataset were obtained using the IQ-TREE web server (Trifinopoulos et al., 2016). The branches support analysis was SH-aLRT (1000 replicates).

2.3 | Genetic distances and standard deviations estimation

Based on the clustering observed in the phylogenetic analyses, the genetic distances and standard deviations, within and between clusters, were estimated using MEGA 7.0 software (Kumar et al., 2016). The 2xSD criterion was used to determine the different genotypes (Chhabra et al., 2019; Kroneman et al., 2013).

2.4 | Bayesian phylodynamic and phylogeographic analyses

A Bayesian analysis was performed with complete ORF2 sequences in order to estimate the evolutionary rate, the time to the most recent common ancestor (tMRCA), and to obtain a maximum clade credibility (MCC) tree. No recombinant sequences (in the ORF2 region) were used. The temporal structure of the dataset was evaluated using TempEst (Rambaut et al., 2016), and the analysis was performed using the Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in BEAST 1.8.4 (Drummond et al., 2012) with BEAGLE library (Ayres et al., 2012) to improve computational time. The TN+I+G, as the best DNA model for the dataset, obtained with IQ-TREE web server (Trifinopoulos et al., 2016), was used as nucleotide substitution model and an uncorrelated relaxed molecular clock model with a lognormal distribution (Drummond et al., 2006). Three tree priors (Constant Size, Exponential Growth, and Bayesian Skyline) were evaluated based on the Akaike's information criterion (AIC) through MCMC (AICM, Baele et al., 2012), and the Bayesian Skyline was selected as the best tree prior. The length of the MCMC chain was 100 million, and the convergence of the analysis was evaluated in Tracer v1.6.0 accepting effective sample size (ESS) values > 200 for all the parameters. The MCC tree was obtained with TreeAnnotator of BEAST 1.8.4 package and visualized with FigTree v1.4.3.

A phylogeographic analysis was performed, in order to reconstruct the evolutionary history of GIII BoNoV. Since there are considerably more partial sequences of the ORF2 than the complete ORF2 in the database, for a more exhaustive analysis, the dataset with the partial sequences was used in order to include as many sequences as possible and with a more widely geographic distribution of these sequences. Briefly, the analysis was performed using the Bayesian MCMC approach implemented in BEAST 1.8.4 (Drummond et al., 2012) with BEAGLE library (Ayres et al., 2012). The TN+I+G was used as nucleotide substitution model, selected as the best DNA model obtained with IQ-TREE web server (Trifinopoulos et al., 2016), and an uncorrelated relaxed molecular clock model with a lognormal distribution (Drummond et al., 2006). The evolutionary rate obtained previously (2.78E-3 substitutions per site per year, s/s/y) was used as a prior. Three tree priors (Constant Size, Exponential Growth, and Bayesian

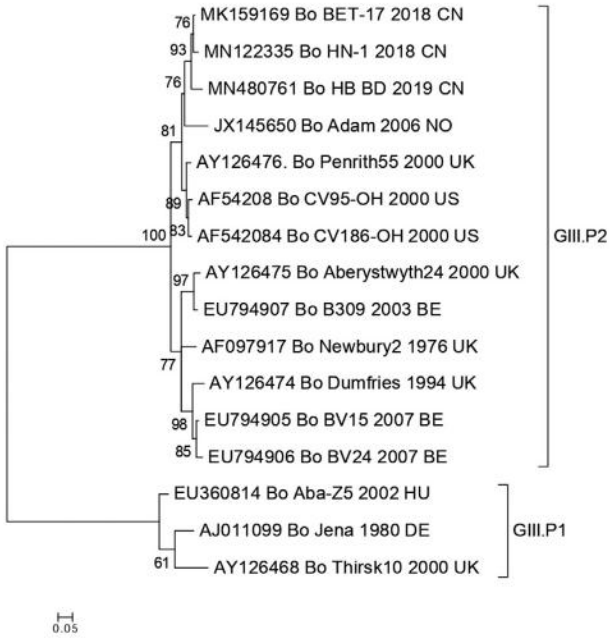

FIGURE 1 Phylogenetic analysis of the RdRp gene. Maximum likelihood tree using the nucleotide sequences of the complete RdRp

Skyline) were evaluated based on the AICM (Baele et al., 2012), and the Bayesian Skyline was selected as the best tree prior. The country of isolation was used as trait for the phylogeographic analysis. The length of the MCMC chain was 100 million, and the convergence of the analysis was evaluated in Tracer v1.6.0 accepting ESS values > 200 for all the parameters. The MCC tree was obtained with TreeAnnotator of BEAST 1.8.4 package and visualized with FigTree v1.4.3. The Bayesian Skyline plot was obtained with Tracer v1.6.0.

Because GIII.2 was the most prevalent genotype, the epidemic history of this subtype was reconstructed, to know in depth the epidemiology of this genotype that has recently emerged. We conducted the analysis with GIII.2 partial ORF2 sequences using a previously described evolutionary rate of 3.81E-3 s/s/y for the complete ORF2 (Mauroy et al., 2014). The other parameters/conditions were similar to the one described previously. The MCC tree was visualized with FigTree v1.4.3 and the Bayesian Skyline plot was obtained with Tracer v1.6.0.

3 | RESULTS

3.1 | Phylogenetic analyses

The phylogenetic analysis using the nucleotide sequences of the complete genomes showed three groups: the GIII.1P[1], the GIII.2P[2], and Chinese sequences between these two groups (Figure S1). In the ML tree using the nucleotide sequences of the complete RdRp, two genotypes were observed: GIII.P1 and GIII.P2 (Figure 1). On the other hand, in the ML tree using the amino acid sequences of the complete capsid, four genotypes were observed: GIII.1–4 (Figure 2).

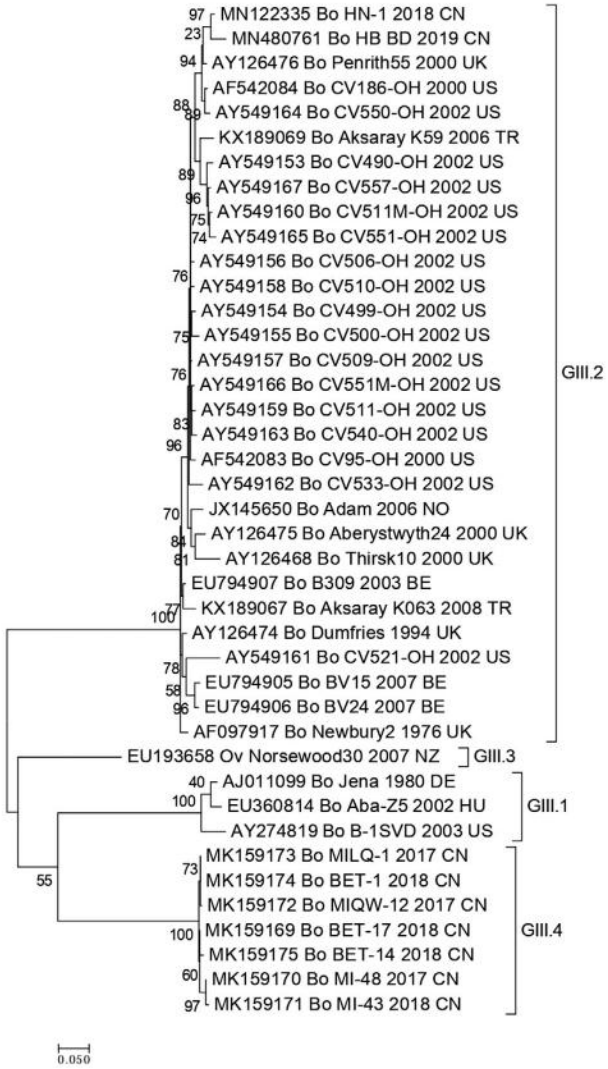

FIGURE 2 Phylogenetic analysis of the capsid. Maximum likelihood tree using the amino acid sequences of the complete capsid

3.2 | Classification of RdRp sequences

In order to confirm the classification in P-types, distances and standard deviations, within and between clusters, were estimated, and the 2xSD criterion was applied. As can be seen in Figure 3a, 2 P-types were confirmed. Unfortunately, the complete RdRp sequence of the strain Norsewood30 is not available and was not included in the analysis.

3.3 | Classification of VP1 sequences

In order to confirm the classification in genotypes, distances and standard deviations, within and between clusters, were estimated, and the 2xSD criterion was applied. As can be seen in Figure 3b, three genotypes were confirmed. Unfortunately, for GIII.3, only one VP1 complete sequence is available, so 2xSD criterion could not be applied as at least two sequences per cluster are required to calculate the standard

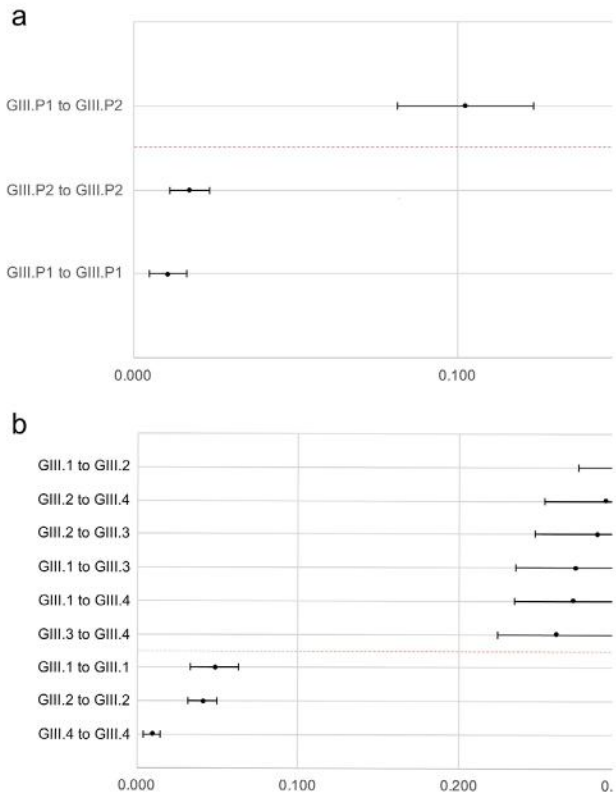

FIGURE 3 P-distance comparison of GIII P-types (a) and genotypes (b). Mean genetic distances are represented with dots and error bars represent 2xsd for each comparison. Y-axes represent comparison of distances within and between P-types (a) and genotypes (b). Below the dotted line, distances within P-type (a) and genotype (b) are indicated and above the dotted line, distances between P-types (a) and genotypes (b) are indicated

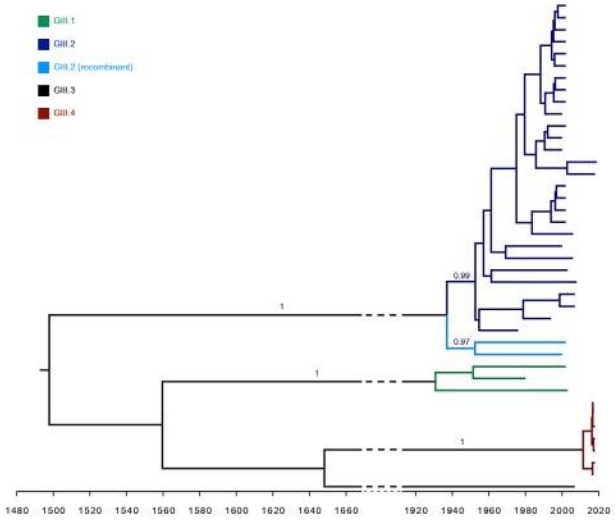

FIGURE 4 Time-resolved Bayesian phylogeny reconstruction with complete ORF2 sequences. A maximum clade credibility tree is shown, obtained with complete ORF2 sequences. Posterior values of key branches are shown. Of note, most of the internal branches were well supported ≥ 0.9 (not shown for clarity)

deviation for within cluster distances. However, in order to maintain coherence with the previous literature, the assignment of the GIII.3 genotype to the ovine norovirus strain Norsewood30 was maintained.

3.4 | Spatiotemporal dynamic of GIII noroviruses

Based on the complete capsid sequences, the time-resolved Bayesian tree reconstruction confirmed the four genotypes observed in the ML tree. Interestingly, the recombinant GIII.2P[1] clustered together and separately from the others genotype GIII.2 sequences, with a common origin few years after the emergence of this genotype (Figure 4). For the genogroup GIII, the evolutionary rate estimated was 2.78E-3 s/s/y (95%HPD, 1.79E-3 s/s/y–3.78E-3 s/s/y) and the tMRCA was estimated around 1500 (95%HPD, 1247–1688). Despite the long history of this genogroup, the genotypes detected at present have a relatively short history, all emerging in the last 100 years. The tMRCAs of each genotype were: for GIII.1 in 1930 (95%HPD, 1898–1953), for GIII.2 in 1937 (95%HPD, 1909–1957), and for GIII.4 in 2011 (95%HPD, 2007–2013). The tMRCA of the recombinant lineage was estimated in 1952 (95%HPD, 1927–1970) (Figure 4). For GIII.3, only one sequence was available so the tMRCA was not estimated with precision. As observed in Figure S2, the demographic analysis showed that these genotypes are in a phase of population reduction.

Based on partial capsid sequences, we observed that the topology of the trees was maintained, with four genotypes (Figure 5). In addition, with more recombinant GIII.2P[1] sequences added to the analysis, we confirmed that all derive from a common ancestor. Although the tMRCA of the genogroup was estimated more recently, around 1700 (95%HPD, 1599–1847), the tMRCA of the genotypes detected at present was consistent, all with a recent emergence (less than 100 years ago). Based on the limited sequences available, the reconstruction of the geographic dispersion of the different genotypes of the genogroup was analyzed through a Bayesian phylogeographic approach (Figure 5). The most probable location of emergence of the genogroup is Uruguay (33%), followed by New Zealand (24%) and Turkey (16%). Uruguay (48%) is the most probable place of emergence of genotype GIII.1, followed by Turkey (20%) and United Kingdom (16%). The emergence of GIII.2 was most probably in United Kingdom (81%), followed by the United States (15%). For GIII.3, there are only two sequences, both from New Zealand, so the most probable origin of this genotype was located in this country. The recently described, and here classified as genotype GIII.4, had it most probable origin of emergence in Uruguay (50%), followed by China (20%) and Turkey (16%).

3.5 | Evolutionary history of GIII.2

As genotype GIII.2 was the most prevalent genotype, its evolutionary history was reconstructed (Figure 6). We confirmed the origin of this genotype in United Kingdom (99% of probability), and from there, it was dispersed worldwide. The recombination event originating a

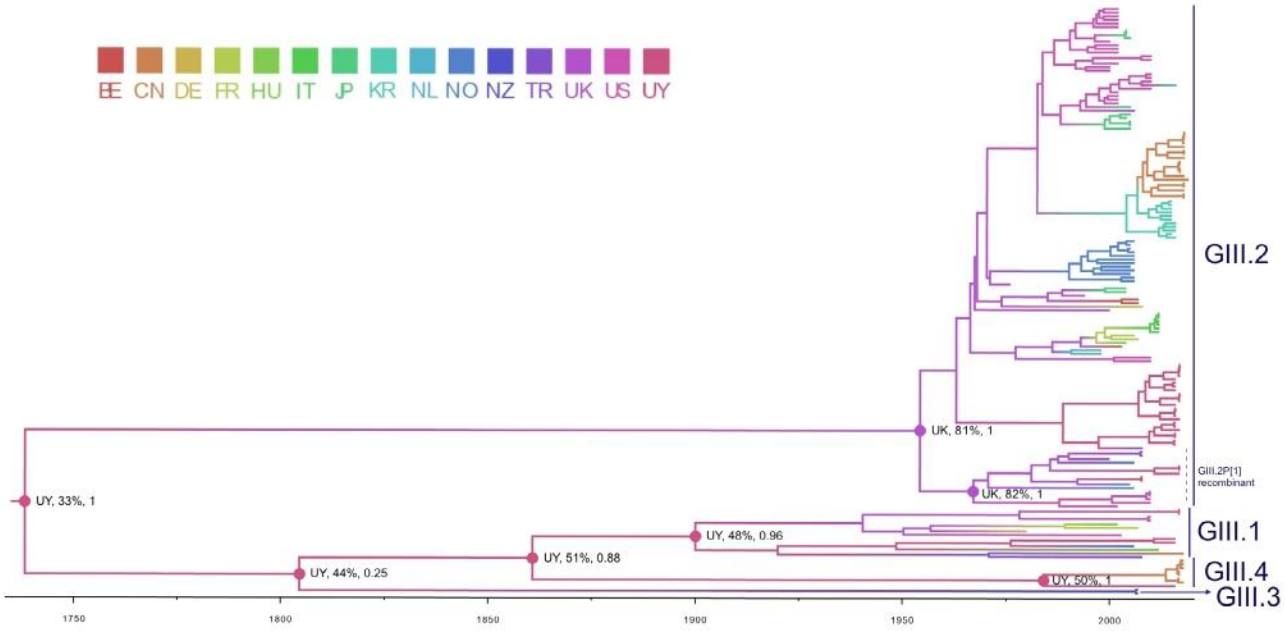

FIGURE 5 Phylogeographic reconstruction of the genogroup GIII. The time-scaled tree is shown, with branches colored by the most probable country (the two-letter country code as defined in ISO 3166-1 alpha-2 was used). In key nodes, the countries (same two-letter code), the probabilities of being the country of origin, and the posterior values are indicated

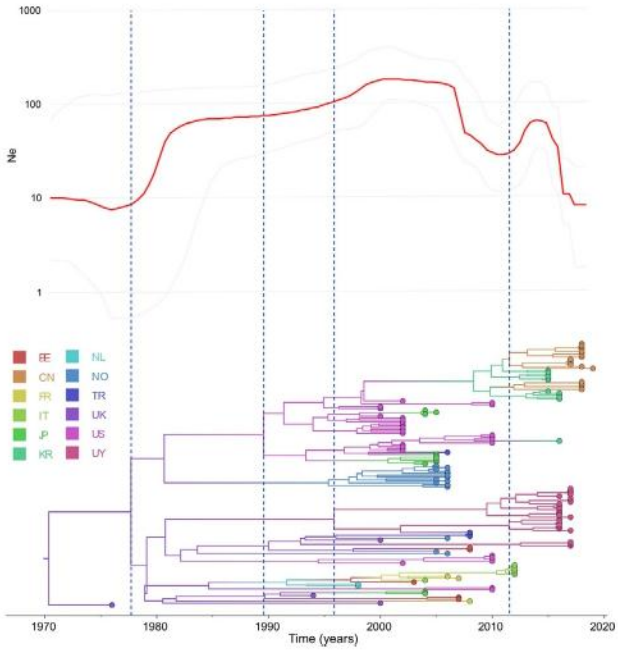

FIGURE 6 Dissemination dynamic of GIII.2 norovirus. The phylogeographic reconstruction of genotype GIII.2, obtained with partial ORF sequences is shown in the lower figure. Branches and nodes are colored by country as indicated in the legend (the two-letter country code as defined in ISO 3166-1 alpha-2 was used). In the upper figure, the Bayesian skyline plot obtained in the same analysis is shown. Ne is the effective number of infections

lineage that then was dispersed worldwide took place in the United Kingdom (95% of probability). Some GIII.2 lineages were dispersed from United Kingdom to other European countries and then arrived to the United States and Japan. Around 1992, a lineage of GIII.2 arrived to the United States and was widely dispersed, arriving Japan, Turkey, and Korea, and from the latter to China. Another lineage arrived to Uruguay from United Kingdom and was widely dispersed in this country, arriving then to Turkey. Interestingly, in the demographic history, we observed three population growths; the first soon after its origin and spread within Europe, the second when it arrived to Uruguay (South America), and the third when it arrived to China (Asia); from 2015, the population began to decline (Figure 6).

4 | DISCUSSION

Recently, an updated classification of norovirus genogroups and genotypes was published (Chhabra et al., 2019); however, this update was focused on human norovirus. On the other hand, several studies have been recently performed in order to characterize norovirus circulating in cattle (Castells et al., 2020; Guo et al., 2018; Karayel-Hacioglu & Alkan, 2019; Mohamed et al., 2017, 2018; Pourasgari et al., 2018; Ryu et al., 2020; Shi et al., 2019; Symes et al., 2018; Turan et al., 2018; Wang et al., 2019). A timely, exhaustive, and complete classification of GIII noroviruses is clearly needed, and in this work, we fill this need. In order to classify the strains within the genogroup GIII, the previously proposed criterion was followed: phylogenetic clustering and 2xSD diversity at amino acid level (Kroneman et al., 2013). In this sense, we determined that GIII could be divided into two P-types (RdRp), GIII.P1 and GIII.P2. Moreover, GIII could be divided into four genotypes (VP1), GIII.1, GIII.2, GIII.3, and GIII.4. On the other hand, as observed in our study, the classification based on complete genomes is intricate, due to the lack of sequences and the recombinant strains (event found mainly

in the joint ORF1-ORF2), so the dual typing proposed previously is also necessary for genogroup III classification.

Interestingly, we confirmed that most of the recombinant GIII.2P[1] strains detected worldwide were originated from the same recombination event around 1950 and whose strain was later dispersed through the world. This scenario is coherent with the clustering of the recombinant strains in the ML and Bayesian phylogenetic analyses. It is important to highlight that the breakpoint of recombination is in the junction of ORF1-ORF2 and for this reason, it is not detected in the region analyzed. Despite some nucleotides of the region analyzed could be product of the recombination, they are few to affect the results obtained.

The evolutionary rate estimated for the genogroup (2.78E-3 s/s/y) is similar to the one estimated previously, 3.81E-3 s/s/y, for the genotype GIII.2 (Mauroy et al., 2014), and usual for a virus with an RNA genome.

The tMRCA estimated in our analysis is consistent with the one obtained previously, around 1500 (Kobayashi et al., 2016), although in the previous study, only one sequence was included in the analysis as an outgroup. Genogroup III has a long history; from the emergence until nowadays, probably different genotypes have emerged and disappeared, and are not detected at present. In this sense, the genotypes detected at the present have emerged in the mid-1900; this was also observed for genotypes of other genogroups, that is, GII.3 and GII.4 (Kobayashi et al., 2016; Motoya et al., 2017; Saito et al., 2020).

Based on the limited sequences available, the emergence of the genogroup probably occurred in Uruguay, but this result should be considered with caution since more strains from geographical distinct locations are needed to establish with more precision the emergence of genogroup. The cattle introduction in Uruguay took place in the beginning of the 17th century (INAC, 2011), but around 1500, the cattle was introduced to the Americas. With the information available, we could not determine the origin of the genogroup; a possible explanation could be that it was originated in South American creole animals, and then jumped to cattle, but the precise origin is still unknown. In Uruguay, a high prevalence of GIII norovirus has been documented (Castells et al., 2020), which could be a predisposing situation for the emergence of norovirus genotypes as probably occurred for GIII.1 and GIII.4.

The reconstruction of the evolutionary history of the genotype GIII.2 showed interesting results. This genotype probably arose in United Kingdom, and then it was dispersed throughout the world, being the most prevalent genotype detected worldwide. In addition, the recombinant lineage also arose in United Kingdom and was also dispersed worldwide. In this sense, the demographic history allowed to understand the dispersion pattern of this genotype. Three population growths were observed and corresponded with the arrival of the genotype to naïve geographic regions, with more susceptible animals, allowing its spreading. The cross-reactivity with heterologous antigen and convalescent antisera from calves inoculated with either GIII.1 or GIII.2 was undetectable or low, determining that they are antigenically distinct (Oliver et al., 2006). This genotype-specific neutralization of norovirus seems to be common, independently of the genogroup (Ford-Siltz et al., 2020). Contrarily to the consecutive growth phases, at present, this genotype is in a phase of population reduction, which could be explained by the reduction in the susceptible population, and/or the recent emergence of the genotype GIII.4, which could be replacing genotypes GIII.1 and GIII.2. This replacement has been widely documented for norovirus, not only at genotype level (Lu et al., 2015), but within genotype, that is, the GII.4 variants replacement (Parra et al., 2017).

The results obtained could guide the vaccine design, and reinforces the need to maintain the molecular epidemiology surveillance in order to detect new genotypes or variants antigenically distinct.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this article.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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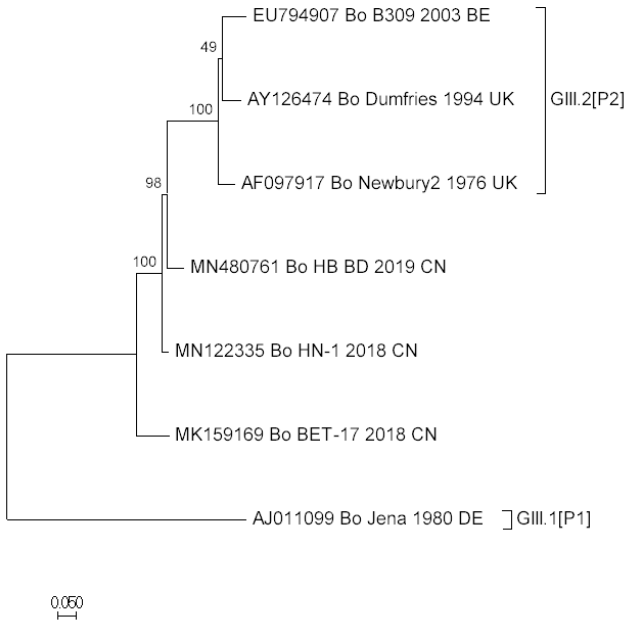

Figure S1. Phylogenetic analysis of complete genomes. The maximum likelihood tree using the nucleotide sequences of the complete genomes available in the database is shown.

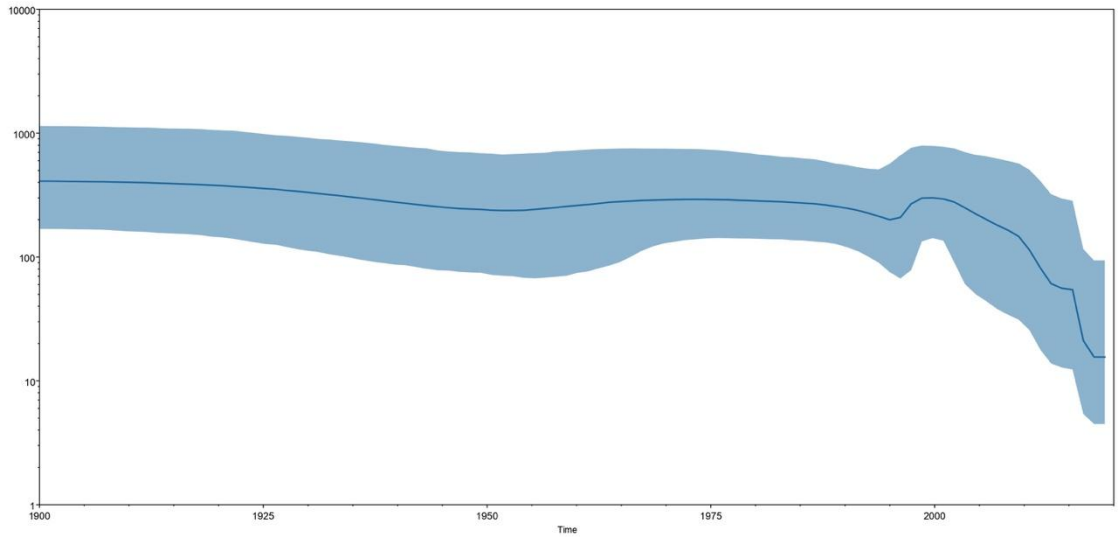

Figure S2. Bayesian skyline plot of the genogroup GIII. The effective number of infections is plotted against the time.

B.6. Estudio de torovirus bovino en muestras clínicas

Artículo 9. Primer reporte y caracterización genética de torovirus bovino en Uruguay

Este reporte tuvo como objetivo reportar por primera vez la presencia de BToV en Uruguay, y el segundo reporte a nivel sudamericano. Además, se caracterizaron genéticamente las cepas detectadas en tres brotes de DNT ocurridos en Uruguay. Se propuso además un sistema de clasificación de BToV a través de análisis filogenéticos y análisis de distancia genética. Con base en esta clasificación, se detectaron dos clados asociados a los brotes, con sustituciones nucleotídicas y aminoacídicas tanto inter como intra brote.

El artículo fue enviado a una revista internacional arbitrada para ser publicado y se encuentra en proceso de revisión por pares.

First report and genetic characterization of bovine torovirus in Uruguay

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Key words: bovine torovirus, calves, Uruguay, diarrhea, genetic diversity

Abstract: Bovine torovirus (BToV) has been described as an enteric pathogen that causes diarrhea in calves and adult cattle, resulting in economic losses due to weight loss and decrease in milk production. The aim of this study was to report the presence and the genetic characterization of BToV in calves in Uruguay. BToV was detected in 42.1% (8/19) of the calves analyzed from 3 diarrhea outbreaks. This is the first documentation of BToV in Uruguay, and the second in South America. We propose a classification of BToV in different clades, based on phylogenetic analysis and genetic distance using a partial S gene region. Based on this classification, we detected two different clades associated to the outbreaks described in this study. Finally, we observed

nucleotide and amino acid substitutions both within and between outbreaks. Further investigation is needed to assess the clinical and economic impact of BToV in this subcontinent.

Introduction

Bovine torovirus (BToV) was first detected in 1979 in a farm near the city of Breda, Iowa, United States (Woode *et al*., 1982); hence it was initially named Breda virus. BToV was allocated into a new family called *Toroviridae* based on the virion's morphology and structure (Horzinek *et al*., 1987). In the current classification by the International Committee on Taxonomy of Viruses, BToV is grouped as: Realm: *Riboviria*, Order: *Nidovirales*, Suborder: *Tornidovirineae*, Familiy: *Tobaniviridae*, Subfamily: *Torovirinae*, Genus: *Torovirus*, Subgenus: *Renitovirus*, Species: *Bovine torovirus* (ICTV, 2018).

The name torovirus comes from the Latin *torus*, due to the shape of the nucleocapsid, which is elongated tubular with helical symmetry and can be bent with the shape of a biconcave or kidney-shaped disk. The viral particles have a peplomeric envelope containing the nucleocapsid (Horzinek *et al*., 1987). The genome is composed of a single stranded RNA molecule of positive polarity and a length of 28,475 nucleotides (Draker *et al*., 2006).

BToV has been described as an enteric pathogen causing mild to profuse, potentially life-threatening diarrhea in calves within the first months of life (Horzinek *et al*., 1987; Hoet and Saif, 2004). Furthermore, it can affect adult cattle causing watery diarrhea and anorexia, somewhat resembling winter dysentery caused by bovine coronavirus (BCoV), which can lead to decrease in milk production in lactating dairy cows (Aita *et al*., 2012). The pathogenicity is mainly due to enteritis, but like coronaviruses, it can also affect the respiratory tract (Hoet el al., 2002; Aita *et al*., 2012). Transmission is mainly via fecal-oral and can be transmitted by the respiratory route (Holmes, 2001).

In South America, BToV has been limitedly studied with only one report in Brazilian diarrheic young and adult cattle (Nogueira *et al*., 2013). The countries members of the MERCOSUR (Argentina, Brazil, Paraguay and Uruguay), are among the 10 principal beef exporters worldwide (USDA, 2020), and Uruguay and Argentina are also dairy leading exporting countries (IDF, 2013). The aim of this study was to determine the presence of BToV and its genomic characterization in calves in Uruguay, broadening the current knowledge on geographic distribution of this virus.

Materials and methods

Samples

Three diarrhea outbreaks (A-C) in dairy farms were studied. Outbreak A, occurred in 2015, and 6 calves were sampled. Outbreak B, occurred also in 2015, and 8 calves were sampled. Outbreak C, occurred in 2017, and 5 calves were sampled. Feces were analyzed from a sample bank stored at -80°C in the Molecular Virology Laboratory of the University of the Republic of Uruguay. Briefly, feces were diluted 1:10 (v:v) in phosphate-buffered saline solution, centrifuged at 3000 g for 20 minutes at 4°C, and the supernatants were obtained.

Viral RNA extraction and reverse transcription

Viral RNA was extracted using QIAamp® cador® Pathogen Mini Kit (Qiagen®), following the manufacturer's instructions, and tested to detect BToV by reverse transcription followed by real time polymerase chain reaction (RT-qPCR). The RT was carried out with RevertAid® Reverse Transcriptase (Thermo Fisher Scientific®) and random hexamers primers (Qiagen®), following manufacturer instructions.

Bovine torovirus screening and ancillary tests

The qPCR was used for screening and was targeted to the nucleocapsid gene as reported elsewhere (Tsuchiaka *et al*., 2016). Briefly, 12.5 μL of SensiFAST™ Probe No-ROX Kit (Bioline ®), 5 μL of DEPC-treated DNAse-free water, 1.0 μL of [10 μM] forward primer, 1.0 μL of [10 μM] reverse primer, 0.5 μL of [10 μM] probe, and 5 μl de cDNA were mixed in each tube and analyzed in a Rotor-Gene Q (Qiagen®). Other viruses were also analyzed as ancillary tests: rotavirus A (RVA), bovine coronavirus (BCoV), bovine norovirus (BoNoV), and bovine astrovirus (BoAstV), as previously reported (Castells *et al*., 2019a; Castells *et al*., 2019b; Castells *et al*., 2020a; Castells *et al*., 2020b).

Conventional PCR

BToV-positive samples were amplified by conventional nested-PCR targeted to the S genomic region, in order to obtain sequences for phylogenetic analysis. Briefly, for the first round of PCR, 5 μL of cDNA, 12.5 μL of MangoMix™ (Bioline®), 4.5 μL of DEPC-treated DNAse-free water, 1.0 μL of dimethyl sulfoxide, and 1.0 μL of [10 μM] forward primer (5'-GTG TTA AGT TTG TGC AAA AAT-3') and 1.0 μL of [10 μM] reverse primer (5'-TGC ATG AAC TCT ATA TGG TGT-3') targeted to the S genomic region described elsewhere (Ito *et al*., 2007). For the second round of PCR, 2 μL of cDNA, 12.5 μL of MangoMix™ (Bioline®), 7.5 μL of DEPC-treated DNAse-free water, 1.0 μL of dimethyl sulfoxide, and 1.0 μL of [10 μM] forward primer (5'- CAG AGG TGC CGT TGT TGT GTC -3') and 1.0 μL of [10 μM] reverse primer (5'- ACA TAG AGC GGT GTC TGT TGA -3') targeted to the S genomic region described elsewhere (Ito *et al*., 2007).

Sequence analyses

The sequences obtained in this study were deposited in GenBank with accession numbers: MW002687-92 and MW002694-95. All the available sequences in GenBank, corresponding to the fragment sequenced in this study

(position 21063-21612, reference sequence AY427798) were downloaded. Multiple sequences alignment was obtained using Clustal W with MEGA 7 software (Kumar *et al*., 2016). Genetic distances were estimated between and within clades with MEGA 7 software. The best model of nucleotide substitution (TN+G4) and the maximum likelihood tree were obtained with W-IQ-TREE (Trifinopoulos *et al*., 2016). The branches support was calculated with 1000 replicas of SH-aLRT test.

Results and discussion

BToV was detected in 8 of the 19 calves sampled (42.1%). By outbreak, BToV was detected in 4 of 6 calves (66.7%) in the outbreak A, 2 of 8 calves (25.0%) in the outbreak B, and 2 of 5 calves (40.0%) in the outbreak C. The detection of BToV in three diarrhea outbreaks, alerts about the circulation of a virus with negative effect in the cattle production in one of the major exporters of beef and dairy products worldwide (IDF, 2013; USDA, 2020).

Notwithstanding BToV has been recognized as a causative agent of diarrhea, the epidemiological data for BToV infection is limited. Clinical signs are similar to those caused by BCoV infections, so it has been suggested that BToV may have been misdiagnosed as BCoV infection by clinical investigations (Aita *et al*., 2012). In addition, other major pathogens are usually detected, attributing to these, as the etiologic agents of the diarrhea outbreaks. Here, we reported the coinfection of BToV with other viruses such as RVA, BCoV, BoNoV, and BoAstV. In outbreak A, in addition to BToV, we detected RVA (6/6), BCoV (3/6), BoNoV (6/6), and BoAstV (1/6). In outbreak B, in addition to BToV, we detected RVA (6/8), BoNoV (7/8), and BoAstV (4/8); BCoV was not detected. Interestingly, in outbreak C, we detected only BToV. Due to the multifactoriality of the NCD, it is difficult to determine the role of each agent at the field level. In addition to viruses, other pathogens are usually involved such as bacteria or protozoa, in addition to other calves' factors, such as the immune

status, age, nutritional status, passive immunity intake, among others. However, the detection of BToV in these outbreaks, indicates that it may have a role, either primary or secondary. The study of this virus in the Southern Cone is further encouraged, given its potential negative impact in cattle production and the economy of the South American countries, and further studies are needed to assess the clinical and economic impact of BToV in South America.

The S gene, and specifically the fragment sequenced in this study is the most abundant in GenBank. Other genes, such as N and M, are very similar between different isolates, being less adequate for genetic classification. In addition, the S gene codifies for the spike protein, a surface protein which determines the antigenicity (Ito *et al*., 2007). There is not a consensus about the classification of BToV, so we decided to use the partial fragment of the S gene mentioned previously. Currently, two serotypes have been described, BRV-1 and BRV-2, and both caused diarrhea in gnotobiotic calves (Woode *et al*., 1985). In addition, the complete genome of serotype 1 has been obtained (Draker *et al*., 2006), but no sequence was available for the serotype 2, which may indicate that is not widely dispersed.

Recently, there were described 4 clades, using 12 complete S genes sequences (Li *et al*., 2020), but AY427798 and AF076621 correspond to the same strain, being AY427798 a more reliable sequence (Draker *et al*., 2006). For this reason, we did not include AF076621 in the classification in different clades. Leaving aside this small difference, we observed that with the complete S gene and with the partial fragment analyzed in our work, the same 3 clades were observed (Figure 1). Despite we consider that classifying BToV would be better using complete S gene sequences, taking into account the comparability of classifying in clades with both regions, the greater ease of amplifying and sequencing a shorter fragment, and that there is a greater number of sequences in GenBank to compare genetically, using the partial fragment is also encouraged.

Phylogenetic analysis showed that Uruguayan strains clustered in two clades, B and C. Uruguayan strains from clade B, clustered close to European strains from Netherlands and Croatia; other strains belonging this clade were from China and South Korea (Figure 1). Uruguayan strains from clade C, clustered in a separate sub-clade; other strains belonging this clade were from Japan (Figure 1). The strains from outbreak A and B, both from 2015, clustered together in clade C, and strains from outbreak C, from 2017, clustered in clade B (Figure 1). There were probably at least two introductions of BToV in Uruguay, but further analysis needs to be done as some clades may have arisen in Uruguay as well. It is important to note that strains belonging to different clades showed relatively low cross-reactivity (Ito *et al*., 2010), which could have implications if a vaccine is developed and/or possible re-infection of calves with BToV strains belonging to different clades.

In addition, we determined the genetic distances between clades to deepen the classification. Within clades, we observed genetic distances lower than 5.0% (Table 1), confirming the genetic closeness between the strains within a clade. By the other hand, genetic distances between different clades were higher than 5.0% (Table 1). Based on this results, the value of 5.0% in genetic distances could be used as threshold to determine different clades. Additionally, we estimated the genetic distance between BToV clades and other related toroviruses, such as goat torovirus, Tasmanian devil torovirus, and equine torovirus (Supplementary table 1). We observed that both goat and Tasmanian devil toroviruses were closely related to BToV, as also observed in the phylogenetic analysis; further studies should be performed to determine if exist the possibility of interspecies transmission.

Interestingly, within outbreak A, we observed a nucleotide substitution A634T in the strain BToV/URY/LVMS669/2015, which also changed the amino acid T212S, and a nucleotide substitution A715C in the strain BToV/URY/LVMS665/2015, which also changed the amino acid T239P. Both

strains within the outbreak B were identical, and both strains within the outbreak C were also identical. Strains from outbreak A and B were similar, with only one nucleotide difference in the position 103 of the alignment, with a cytosine in the outbreak A and a guanine in the outbreak B, and this nucleotide substitution changed the amino acid, being glutamine in the outbreak A and glutamic acid in the outbreak B. These results alert about the continuous substitutions in BToV, not only between different outbreaks in the same year but also substitutions within an outbreak, with its possible antigenic drift, as observed for a related virus, BCoV, where a single amino acid change in the spike protein conferred resistance to virus neutralization (Yoo and Deregt, 2001).

Conclusions

Bovine torovirus was detected in 3 diarrhea outbreaks in Uruguay. Despite its role in the outbreak was not determined, it may play a primary or a secondary role, but in both scenarios with a negative impact in cattle production. We detected the circulation of 2 different clades, and both nucleotide and amino acid substitutions within and between outbreaks, with possible implications related to immune escape. In addition, we determined the threshold in the genetic distances to classify BToV in clades.

Declarations

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Research involving Human Participants and/or Animals: Animal welfare was taken care of, since no invasive procedure was used.

Informed consent: Not applicable

Conflicts of interest: None.

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Table 1. Genetic distances within and between clades.

Clades	Distance ± standard error
A	3.16 ± 0.40
B	3.35 ± 0.43
C	4.24 ± 0.54
A vs. B	8.71 ± 1.10
A vs. C	9.54 ± 1.17
B vs. C	6.58 ± 0.86

Supplementary table 1. Genetic distances between BToV clades and other related torovirus.

Clades compared		Distance ± standard error
B	C	**6.58 ± 0.86**
C	Tasmanian devil	8.07 ± 1.17
A	B	**8.71 ± 1.10**
B	Tasmanian devil	8.83 ± 1.31
A	Tasmanian devil	9.27 ± 1.33
A	C	**9.54 ± 1.17**
Goat	Tasmanian devil	9.68 ± 1.50
C	Goat	10.12 ± 1.37
B	Goat	10.49 ± 1.52
A	Goat	12.36 ± 1.57
Tasmanian devil	Equine	34.35 ± 3.23
Goat	Equine	34.40 ± 3.29
C	Equine	35.01 ± 3.24
B	Equine	35.16 ± 3.31
A	Equine	38.30 ± 3.50

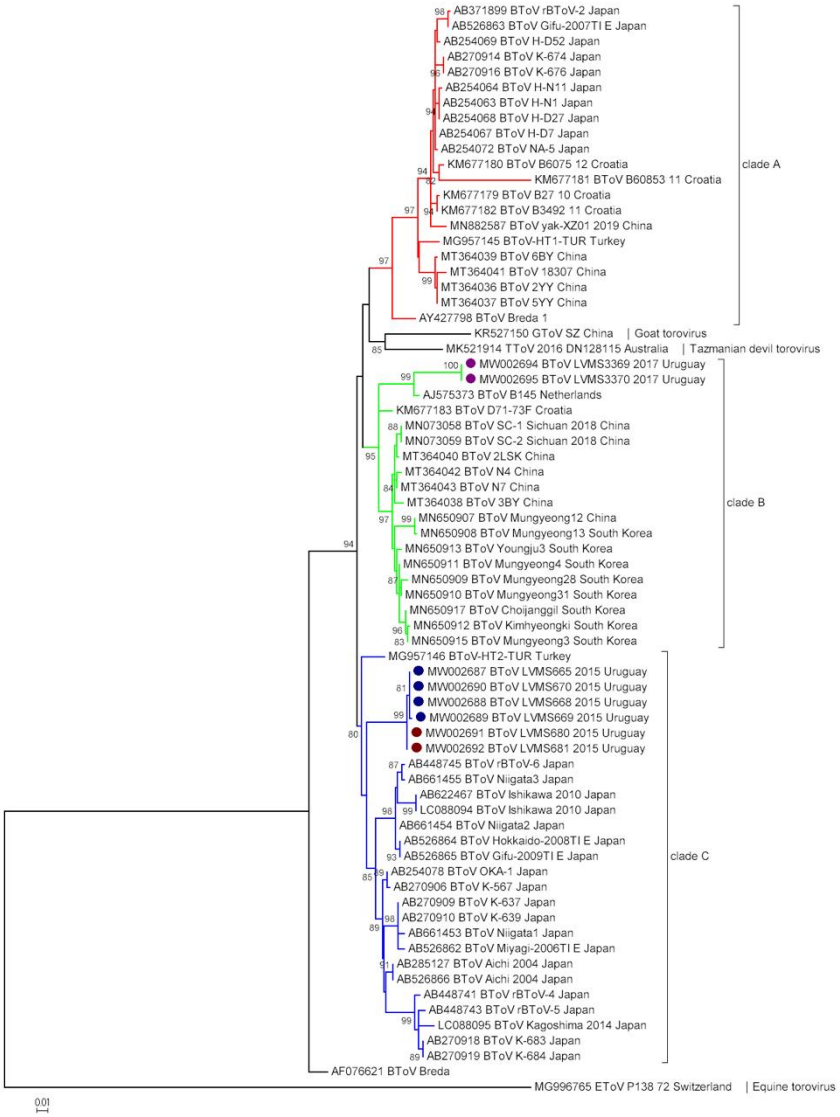

Figure 1. Phylogenetic analysis. A maximum likelihood tree is shown with the Uruguayan strains obtained in this work indicated with blue, red, and violet filled circles for Outbreak A, B and C strains, respectively. aLRT values ≥ 80 are shown in each node.

Artículo 10. Frecuencia de detección y factores de riesgo asociados a la infección por torovirus bovino en Uruguay

Este reporte está dirigido a reportar la presencia de torovirus bovino, determinando la frecuencia de infección de este virus en terneros en Uruguay. Se comparó la frecuencia de detección en diferentes grupos, determinando de esta manera posibles factores de riesgo que pueden facilitar la infección por este virus.

La frecuencia de detección fue de 8% (22/278), y fue detectado únicamente en terneros lecheros y no en terneros de carne. El virus fue detectado tanto en terneros con diarrea como en terneros sin diarrea. En cuanto a la distribución geográfica, fue detectado principalmente en Colonia, seguido por Soriano, los únicos dos departamentos con casos positivos. Por otro lado, fue detectado también en casos de terneros naturalmente muertos con diarrea como posible causa de muerte.

Este reporte, se encuentra aún en proceso de escritura, análisis, y restan incluir algunos resultados para darle mayor soporte estadístico a las observaciones. De todas formas, se incluye el borrador inicial, ya que los datos obtenidos hasta el momento resultan novedosos y relevantes a nivel país. El trabajo no fue enviado aún a una revista internacional arbitrada, lo cual se realizará al completar los análisis.

Frequency of detection and risk factors associated with bovine torovirus infection

Abstract: Bovine torovirus (BToV) has been described as an enteric pathogen that causes diarrhea in calves and adult cattle, resulting in economic losses due to weight loss and decrease in milk production. The aim of this study was to determine the frequency of BToV infection in calves in Uruguay. BToV was detected in 7.9% (22/278) of fecal samples, and was detected in dairy but not in beef calves. BToV was detected in diarrheic and non-diarrheic calves. However, differences in BToV detection were observed by year of sampling and geographic location.

Bovine torovirus (BToV) was first detected in 1979 (Woode *et al*., 1982) in a farm near the city of Breda, Iowa, United States; hence it was initially named *Breda virus*. BToV was allocated into a new family called *Toroviridae* based on the virion's morphology and structure (Horzinek *et al*., 1987). In the current classification by the International Committee on Taxonomy of Viruses, BToV is grouped as: Realm: *Riboviria*, Order: *Nidovirales*, Suborder: *Tornidovirineae*, Familiy: *Tobaniviridae*, Subfamily: *Torovirinae*, Genus: *Torovirus*, Subgenus: *Renitovirus*, Species: *Bovine torovirus* (ICTV, 2018).

The name torovirus comes from the Latin *torus*, due to the shape of the nucleocapsid, which is elongated tubular with helical symmetry and can be bent with the shape of a biconcave or kidney-shaped disk. The viral particles have a peplomeric envelope containing the nucleocapsid (Horzinek *et al*., 1987). The genome is composed of a single stranded RNA molecule of positive polarity and a length of 28,475 nucleotides (Draker *et al*., 2006).

BToV has been described as an enteric pathogen causing mild to profuse, potentially life-threatening diarrhea in calves within the first months of life (Hoet and Saif, 2004; Horzinek *et al*., 1987). Furthermore, it can affect adult cattle causing watery diarrhea and anorexia, somewhat resembling winter dysentery caused by bovine coronavirus (BCoV), which can lead to decrease in milk production in lactating dairy cows (Aita *et al*., 2012). The pathogenicity is mainly due to enteritis, but like coronaviruses, it can also affect the respiratory tract (Aita *et al*., 2012; Hoet el al., 2002). Transmission is mainly via fecal-oral and can be transmitted by the respiratory route (Holmes, 2001).

In South America, to our knowledge, BToV has been reported in Brazil (Nogueira *et al*., 2013), and recently by our group in Uruguay (Castells and Colina, unpublished). The aim of this study was to determine the frequency of BToV infection in calves in Uruguay and the risk factors associated with this infection, broadening the current knowledge about BToV in the South America.

To fulfill this purpose, 278 fecal samples were collected from calves from dairy and beef herds. Feces were diluted 1:10 (v:v) in phosphate-buffered saline

solution, centrifuged at 3000 g for 20 minutes at 4°C, and the supernatants were obtained. Viral RNA was extracted using QIAamp® cador® Pathogen Mini Kit (Qiagen®), following the manufacturer's instructions, and tested to detect BToV by reverse transcription followed by real time polymerase chain reaction (RT-qPCR). The RT was carried out with RevertAid® Reverse Transcriptase (Thermo Fisher Scientific®) and random hexamers primers (Qiagen®), following manufacturer instructions. The qPCR was targeted to the nucleocapsid gene as reported elsewhere (Tsuchiaka *et al*., 2016). Categorical data was evaluated with jamovi software (available at https://www.jamovi.org/), through Chi-square tests, and differences were considered statistically significant if the obtained p-value was <0.05.

The overall frequency of BToV detection was 7.9% (22/278) (Figure 1a). BToV has been detected in several countries, with a frequency ranging from 1.1% to 43.2% (Cho *et al*., 2013; Lojkić *et al*., 2015). In Brazil, a bordering country, the frequency observed was similar (6.25%) to the one reported in our study (Nogueira *et al*., 2013). When we analyzed separately by cattle exploitation types, BToV was only detected in dairy (9.2%, 22/239) (Figure 1b), but not beef (0.0%, 0/39) (Figure 1c) calves; this difference was statistically significant ($p<0.05$). To our knowledge this is the first study comparing BToV detection in dairy versus beef calves in the same country. A limitation of this study was the unequal sampling of dairy and beef calves.

We did not observe differences between the proportion of BToV detection in diarrheic (14%, 6/43) and non-diarrheic (13.2%, 5/38) calves (Figure 1d) ($p=0.93$). Unfortunately, for 197 samples this information was unavailable. Other studies have demonstrated that BToV is more often detected in diarrheic than non-diarrheic calves (Haschek *et al*., 2005; Ito *et al*., 2007; Kirisawa *et al*., 2007; Cho *et al*., 2013). Further studies are needed to assess the clinical and economic impact of BToV in South America.

Samples were collected over a four-year period (2015-2018); BToV was detected in samples collected in 2015 (25.9%, 7/27), 2016 (12.6%, 11/87), 2017 (3.3%, 4/122), and was not detected in samples from 2018 (0.0%, 0/42) (Figure 1e). Frequency decreased through time, and this decline was adjusted by a logarithmic regression with an $R^2=0.99$. Differences between years were statistically significant ($p<0.05$), except between 2015 and 2018 ($p<0.1$), and between 2017 and 2018 ($p=0.2$). In addition, we obtained samples from 8 of the 19 departments of Uruguay. The geographic information was available for 16 (72.7%) of the 22 BToV-positive calves, which were from Colonia (93.8%, 15/16) and Soriano (6.2%, 1/16). BToV was not detected in the other 6 departments sampled (Cerro Largo, Florida, Río Negro, Rocha, San José, and Tacuarembó), although, as stated above, this information was not available for 6 of the BToV-positive calves. The frequency of BToV detection was 34.9% (15/43) in Colonia and 9.1% (1/11) in Soriano. Statistically, Colonia showed higher frequency than the other departments ($p<0.05$), except Soriano ($p<0.1$). At herd level, of 5 herds

from Colonia included in this study, 4 (80.0%) had at least one BToV-positive calf. Surveillance should be maintained to detect possible factors influencing BToV circulation.

In order to compare the frequency of BToV detection in live and deceased calves, 47 samples of intestinal contents from deceased calves were also analyzed. Intestinal contents were analyzed as previously detailed for fecal samples. BToV was detected with a higher frequency in deceased (14.9%, 7/47) (Figure 1f) than live calves (Figure 1a); this difference was not statistically significant (p=0.12).

Notwithstanding BToV has been recognized as a causative agent of diarrhea, the epidemiological data for BToV infection is limited. Clinical signs are similar to those caused by BCoV infections, so it has been suggested that BToV may have been misdiagnosed as BCoV infection by clinical investigations (Aita *et al*., 2012). Besides the clinical similarities between both viruses, in Uruguay, BToV and BCoV showed epidemiological similarities too, with similar frequency of detection (7.9% and 7.8%, respectively), were detected in similar proportions in diarrheic and non-diarrheic calves, and in deceased and live calves (Castells *et al*., 2019).

The countries members of the MERCOSUR (Argentina, Brazil, Paraguay and Uruguay), are among the 10 principal beef exporters worldwide (USDA, 2020), and Uruguay and Argentina are dairy leading exporting countries too (IDF, 2013). However, BToV has been limitedly studied in South America, with only one report in Brazilian diarrheic young and adult cattle (Nogueira *et al*., 2013), and one in Uruguay (Castells and Colina, unpublished). The study of this virus in the Southern Cone is further encouraged, given its potential negative impact in cattle production and the economy of the South American countries.

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Conflicts of interest: None.

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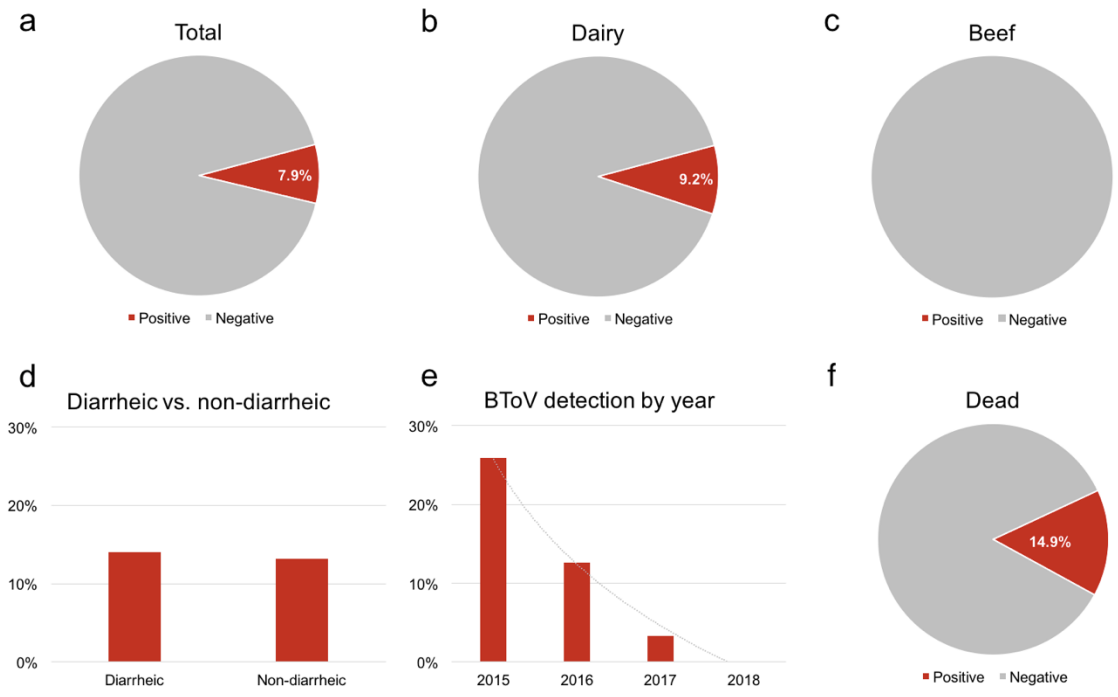

Figure 1. Bovine torovirus detection. a) Overall BToV detection. b) BToV detection in dairy calves. c) BToV detection in beef calves. d) BToV detection in diarrheic and non-diarrheic calves. e) BToV detection by year of sampling. f) BToV detection in naturally deceased calves.

B.7. Anexo

Artículo 11. Causas de diarrea neonatal de terneros y mortalidad en tambos a base de pastura en Uruguay: un estudio de casos y controles

Este artículo abordó el estudio genérico del síndrome de diarrea neonatal (SDN), a través de un estudio de casos y controles, donde fueron incluidos resultados sobre RVA, BCoV y BoAstV, análisis en los cuales tuve participación activa, y se relacionan con la tesis. Al tratarse de un artículo relacionado a la tesis, pero no ser parte fundamental de la misma, solamente se muestra el resumen y la cita para quien desee ahondar en el tema: Caffarena RD, Casaux ML, Schild CO, Fraga M, **Castells M**, Colina R, Maya L, Corbellini LG, Riet-Correa F, Giannitti F. Causes of neonatal calf diarrhea and mortality in pasture-based dairy herds in Uruguay: a farm-matched case-control study. Braz J Microbiol. 2021 Feb 11. doi: 10.1007/s42770-021-00440-3.

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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*

Artículo 12. Efecto de la administración de cepas de *Lactobacillus spp.* sobre la diarrea neonatal, parámetros inmunológicos y abundancia de patógenos en terneros pre-destete.

Este artículo abordó el efecto de dos posibles probióticos sobre la diarrea neonatal. Se estudió entre otros aspectos, el efecto de estos probióticos sobre la infección por RVA y BCoV, análisis en los cuales participé activamente y se encuentran relacionados con esta tesis. Al tratarse de un artículo relacionado a la tesis, pero no ser parte fundamental de la misma, solamente se muestra el resumen y la cita para quien desee ahondar en el tema: Fernández S, Fraga M, **Castells M**, Colina R, Zunino P. Effect of the administration of *Lactobacillus spp.* strains on neonatal diarrhoea, immune parameters and pathogen abundance in pre-weaned calves. Benef Microbes. 2020; 11(5): 477-488. doi: 10.3920/BM2019.0167.

Beneficial Microbes, 2020; 11(5): 477-488

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Effect of the administration of *Lactobacillus* spp. strains on neonatal diarrhoea, immune parameters and pathogen abundance in pre-weaned calves

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RESEARCH ARTICLE

Abstract

Neonatal calf diarrhoea is one of the challenges faced by intensive farming, and probiotics are considered a promising approach to improve calves' health. The objective of this study was to evaluate the effect of potential probiotic lactobacilli on new-born dairy calves' growth, diarrhoea incidence, faecal score, cytokine expression in blood cells, immunoglobulin A (IgA) levels in plasma and faeces, and pathogen abundance in faeces. Two *in vivo* assays were conducted at the same farm in two annual calving seasons. Treated calves received one daily dose of the selected lactobacilli (*Lactobacillus reuteri* TP1.3B or *Lactobacillus johnsonii* TP1.6) for 10 consecutive days. A faecal score was recorded daily, average daily gain (ADG) was calculated, and blood and faeces samples were collected. Pathogen abundance was analysed by absolute qPCR in faeces using primers directed at *Salmonella enterica*, rotavirus, coronavirus, *Cryptosporidium parvum* and three *Escherichia coli* virulence genes (*eae*, *clpG* and *Stx1*). The faecal score was positively affected by the administration of both lactobacilli strains, and diarrhoea incidence was significantly lower in treated calves. No differences were found regarding ADG, cytokine expression, IgA levels and pathogen abundance. Our findings showed that oral administration of these strains could improve gastrointestinal health, but results could vary depending on the calving season, which may be related to pathogen seasonality and other environmental effects.

Artículo 13. Enfermedades asociadas con el virus de la diarrea viral bovina subtipos 1a y 2b en ganado de carne y leche en Uruguay

Este artículo sobre las enfermedades asociadas con el virus de la diarrea viral bovina (DVB) incluyó análisis para RVA y BCoV, como posibles agentes etiológicos de enfermedades asociadas a DVB, los cuáles fueron analizados dentro del marco de esta tesis, por lo que tuve participación activa en el trabajo. Al tratarse de un artículo relacionado a la tesis, pero no ser parte fundamental de la misma, solamente se muestra el resumen y la cita para quien desee ahondar en el tema: da Silva Silveira C, Maya L, Casaux ML, Schild C, Caffarena D, Aráoz V, da Costa RA, Macías-Rioseco M, Perdomo Y, **Castells M**, Colina R, Fraga M, Riet-Correa F, Giannitti F. Diseases associated with bovine viral diarrhea virus subtypes 1a and 2b in beef and dairy cattle in Uruguay. Braz J Microbiol. 2019 Oct 24. doi:10.1007/s42770-019-00170-7.

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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.

Artículo 14. Encefalitis asociada a astrovirus bovino neurotrópico, ¿una enfermedad subdiagnosticada en Sudamérica?

En este artículo describimos un segundo caso de muerte por encefalitis asociada a una infección por astrovirus. Para la detección se utilizó la metodología puesta a punto durante el desarrollo de esta tesis. Tuve una participación activa en el trabajo, detectando el virus en una muestra de cerebro de una vaca lechera del departamento de San José y caracterizándolo filogenéticamente. Al tratarse de un artículo relacionado a la tesis, pero no ser parte fundamental de la misma, solamente se muestra el resumen para quien desee ahondar en el tema. El artículo fue aceptado para su publicación en la Revista Argentina de Microbiología: Doncel Díaz B, **Castells M**, Maya L, Fraga M, Uzal FA, Colina R, Giannitti F. Neurotropic bovine astrovirus-associated encephalitis: An underdiagnosed disease in South America? Rev Argent Microbiol. 2021 (aceptado).

Encefalitis asociada a astrovirus bovino neurotrópico, ¿una enfermedad subdiagnosticada en Sudamérica?

Resumen
Describimos un caso de encefalitis asociada a infección por astrovirus bovino neurotrópico en una vaca lechera, raza Jersey, del departamento de San José, Uruguay. Este representa el segundo caso reportado de esta condición en el hemisferio sur. La vaca, única afectada de un rodeo de 70 bovinos, manifestó signos clínicos neurológicos con curso de 2 días, luego de los que murió espontáneamente. El examen histopatológico reveló meningoencefalitis linfocítica, histiocítica y plasmacítica, con necrosis neuronal, sin cuerpos de inclusión. No se detectaron en el cerebro otras etiologías infecciosas, entre ellas, virus de la rabia (*Lyssavirus*), alfaherpesvirus bovino-1 y alfaherpesvirus bovino-5 (*Varicellovirus*), virus de la diarrea viral bovina (*Pestivirus*), virus del Nilo Occidental (*Flavivirus*), *Listeria monocytogenes*, *Histophilus somni* y otras bacterias. Dado que el descubrimiento de astrovirus neurotrópicos en varias especies de mamíferos, incluidos humanos, es reciente, proponemos que los casos de encefalitis por astrovirus pudieron haber pasado inadvertidos en Sudamérica. Discutimos brevemente el diagnóstico patológico diferencial de encefalitis infecciosas en bovinos.

Neurotropic bovine astrovirus-associated encephalitis: An underdiagnosed disease in South America?

Abstract

We describe a case of neurotropic bovine astrovirus-associated encephalitis in a Jersey dairy cow from the department of San José, Uruguay. This represents the second case of this condition reported in the Southern Hemisphere. The cow was the only one affected in a herd of 70 cows, showing neurological signs with a 2-day clinical course, before dying spontaneously. Histopathological examination revealed lymphocytic, histiocytic, and plasmacytic meningoencephalitis with neuronal necrosis, without detectable inclusión bodies. Other infectious etiologies, including *Rabies virus* (*Lyssavirus*), *Bovine alphaherpesvirus-1* and *Bovine alphaherpesvirus-5* (*Varicellovirus*), *Bovine viral diarrea virus* (*Pestivirus*), *West Nile virus* (*Flavivirus*), *Listeria monocytogenes*, *Histophilus somni* and other bacteria, were not detected in the brain. We propose that given the recent discovery of neurotropic astroviruses in various mammalian species, including humans, cases of astrovirus encephalitis may have gone undetected in South America. We briefly discuss the differential pathologic diagnosis of infectious bovine encephalitis.

Artículo 15. Desarrollo de un producto basado en IgY para prevenir la diarrea por coronavirus bovino

Este artículo surgió de la colaboración entre el Laboratorio de Virología Molecular, al cual pertenezco, y el Instituto de Virología de INTA-Castelar, Argentina, al cual pertenece mi cotutora Viviana Parreño. Los autores del trabajo somos: Marina Bok, Celina Vega, **Matías Castells**, Rodney Colina, Andres Wigdorovitz y Viviana Parreño. Más allá de este artículo se generó una red internacional del estudio de patógenos asociados a DNT, con investigadores de Uruguay, Argentina y Brasil, la cual permitirá avanzar en el conocimiento a nivel regional de estos patógenos que afectan la producción ganadera, base fundamental de la economía de los países mencionados. Al tratarse de un artículo anexo a la tesis, y no ser el primer autor, no se presentan los resultados en detalle; los mismos estarán disponibles al momento de publicarse el artículo.

Developing an IgY-based product to prevent bovine Coronavirus diarrhea

Abstract

Bovine Coronavirus (BCoV) is a major pathogen associated with diarrhea that affects calves. To prevent BCoV diarrhea, calves need to suck maternal colostrum within the first six to twelve hours of life before gut closure. Although there are vaccines to control BCoV diarrhea, there is a need to develop new strategies to complement prevention and treat BCoV diarrhea, where IgY technology represents a promissory tool. In this work, we standardized IgY Antibodies (Abs) production on an industrial scale and validate assays associated with potency quality control. Two hundred laying hens were immunized with semi-purified BCoV to obtain spray-dried powder enriched in specific IgY Abs to BCoV. To measure IgY to BCoV titers in product batches, ELISA assays were statistically validated. With a sample size of 241, IgY to BCoV ELISA showed a sensitivity and specificity of 97,7% and 98,2% respectively. To evaluate the specificity of IgY Abs to BCoV, we run in vitro virus neutralization tests. Finally, a pilot study showed a delay and reduction of BCoV infection and diarrhea with the administration of 40 g of egg powder containing a final IgY Ab titer to BCoV of 512 to newborn calves during 14 days as passive treatment. To our knowledge, this is the first work describing the industrial production of a product based on egg powder to prevent BCoV-associated neonatal calf diarrhea.

5. Discusión general

Esta tesis tuvo como objetivo caracterizar a los virus que afectan a los bovinos a nivel entérico, principalmente en los primeros meses de vida, cuando su impacto negativo es mayor. Para cumplir con este objetivo, se abordó el estudio mediante un enfoque global, caracterizándolos a nivel clínico, pero también determinando el grado de contaminación que tienen las aguas que se utilizan para que consuman los terneros.

A nivel ambiental, se utilizó RVA como indicador de contaminación viral (Castells *et al*., 2018). En cuanto a los resultados obtenidos sobre la contaminación en las aguas de consumo de los terneros en los tambos, se comprobó que las mismas están contaminadas con RVA (35%). Como era de esperar, las aguas de los bebederos (44%) estaban más contaminadas que las provenientes directamente de los pozos (20%). Esto se debe a que las aguas tomadas de los bebederos tienen la contaminación por ser agua de pozo, pero se le suma la contaminación directa por heces de terneros infectados. Se comprobó además, la presencia de partículas infectivas de RVA, mediante aislamiento y detección por inmunofluorescencia del virus, luego de pasajes por cultivo celular. La presencia de virus con capacidad infectiva en el agua, explicaría, al menos en parte, el origen de brotes de diarrea en establecimientos donde se tiene un buen manejo del rodeo, pero no se considera la posibilidad de contaminación ambiental. Esto se comprobó con un caso particular, en el cual se detectó la presencia de RVA en el agua, antes del inicio de un brote de diarrea a causa de este virus en un tambo. Desafortunadamente, no se pudo obtener la secuencia de la/s cepa/s detectada/s en el agua, de manera de confirmar que efectivamente se trató de la misma cepa que generó el brote. Además, se observó un patrón de estacionalidad, con mayor frecuencia de detección en los meses más fríos con respecto a los más cálidos. Por otro lado, no se observó diferencias en la frecuencia de detección de RVA con respecto al tamaño del rodeo del establecimiento (Castells *et al*., 2018).

De manera fundamental para el progreso de la tesis, fue el desarrollo de metodologías moleculares para la detección y caracterización molecular de RVA bovino, BCoV, BoNoV, BToV y BoAstV. Fueron evaluadas diversas metodologías, optimizando cada una de ellas a las condiciones de nuestro laboratorio, obteniendo como resultado metodologías sumamente sensibles y específicas para los cinco virus estudiados (Castells *et al*., 2019a-b; Giannitti *et al*., 2019; Castells *et al*., 2020a-b; Castells *et al*., 2021).

Conociendo y considerando el grado de contaminación ambiental, esta tesis continuó con el estudio del impacto que tienen los virus, pero a nivel clínico. Se estudiaron los cinco virus mencionados previamente. Algunos tienen un reconocido rol en la DNT y están ampliamente estudiados: RVA y BCoV, otros para los cuales se ha demostrado su rol como agente causal de DNT, pero

hay pocos estudios a nivel de campo: BoNoV y BToV, y por último se estudió BoAstV, un virus que aún no está demostrado su rol como agente causal de DNT, pero su estudio está emergiendo, principalmente por su capacidad de infectar a nivel extra-intestinal.

Los cinco virus estudiados fueron detectados, con frecuencias de 66%, 57%, 26%, 9%, y 8%, para BoNoV, RVA, BoAstV, BToV, y BCoV, respectivamente (Castells *et al*., 2019a-b; Castells *et al*., 2020a-c). Llamativamente, BoNoV fue el virus con mayor circulación en terneros en Uruguay, incluso mayor que RVA, el cual a nivel mundial se considera el más prevalente. Este resultado, alerta sobre la circulación de un virus el cual ha sido desestimado, pero que quizás tenga mayor impacto como agente causal de diarrea neonatal de terneros a nivel mundial, que se irá dilucidando a medida que se estudie con mayor profundidad. Otro resultado interesante, fue la presencia de BoAstV, el cuál además fue detectado como el agente causal de muerte de un novillo y una vaca, ambos por encefalitis (Gianitti *et al*., 2019; Doncel Díaz *et al*., 2021).

A nivel general, se observó que los terneros en las primeras 3 semanas de vida se ven mayormente afectados por los cinco virus, más que aquellos terneros mayores de 3 semanas de edad. Además, con excepción de BToV, los otros cuatro virus fueron detectados tanto en terneros lecheros como terneros para producción de carne; BToV fue solamente detectado en terneros lecheros. No se observaron diferencias significativas entre muestras provenientes de terneros vivos y muestras de terneros muertos (Castells *et al*., 2019a-b; Castells *et al*., 2020a-c). Para BCoV y RVA, se cuenta con vacunas preventivas. En este sentido, si bien es una estrategia utilizada para prevenir la DNT debido a ambos virus, se observó que la vacunación es únicamente efectiva para BCoV, mientras que no lo es para prevenir la infección por RVA (Castells *et al*., 2019b; Castells *et al*., 2020b).

Además, la considerable circulación viral observada, se asocia también con una gran diversidad genética en los virus detectados.

En el caso de BCoV, si bien existe un único serotipo, es decir, a nivel antigénico las cepas están íntimamente relacionadas, a nivel filogenético se observó que las cepas que circulan en Uruguay son divergentes a la cepa vacunal. En este sentido, las cepas uruguayas son similares a cepas argentinas, para las cuales se estudió la neutralización cruzada, obteniendo como resultado que existe neutralización por los anticuerpos generados por la cepa vacunal; de todas maneras, este fenómeno de distanciamiento genético pone de manifiesto la existencia de un proceso evolutivo que, en algún momento, podría dar origen a serotipos emergentes, antigénicamente diferentes, y resalta la importancia de mantener una vigilancia continua sobre este virus para detectar a tiempo si alguna cepa escapa a la protección que confiere hasta el momento la vacuna. Se observó además, que las variantes del virus que circulan actualmente, son dos, e ingresaron desde los países limítrofes, Argentina y Brasil (Castells *et al*.,

2019b). A nivel evolutivo, se observó un sesgo en el uso de codones. El virus utiliza preferentemente aquellos codones que son menos utilizados por el hospedero, evitando competir con la traducción de los genes propios del hospedero. La mayoría de los codones más frecuentes terminan en U. El contenido de G + C y la composición de dinucleótidos influyen en el patrón general de uso de codones de BCoV. Además, el dinucleótido CpG se encuentra subrepresentado, lo que podría deberse a sus propiedades inmunoestimuladoras como el reconocimiento de CpG no metilado por el receptor 9 tipo Toll (TLR9) (Castells *et al*., 2017).

En el caso de RVA, también a nivel filogenético, se observó que las cepas uruguayas tienen un patrón realmente complejo. Se evidenciaron múltiples eventos de reordenamientos génicos, así como también múltiples eventos de transmisión interespecie, incluidos eventos zoonóticos. A nivel de VP4 y VP7, se observó estrecha relación con cepas argentinas, y no brasileñas, lo cual podría ser explicado por la diferencia en las razas ganaderas criadas, ya que en Uruguay y Argentina se crían principalmente *Bos taurus*, mientras que en Brasil se crían mayormente *Bos indicus*, y se ha demostrado que distintas subpoblaciones pueden tener diferente susceptibilidad a distintas variantes genéticas de RVA. En cuanto a los otros genes, VP6 y NSP1-5, lo más destacable es la evidencia de eventos de transmisión interespecie, lo cual era de esperar, ya que en nuestro país es muy común la cría de distintas especies en un mismo establecimiento (donde también conviven humanos), principalmente bovinos, ovinos y equinos, pero también caprinos, suinos, entre otros, y la transmisión interespecie en RVA está ampliamente documentada. Otro resultado interesante, fue la detección de un genotipo raro G24P[33] y T9 (para el gen NSP3) para el cual existe un único reporte a nivel mundial, de una vaca en Japón. Sin embargo, a nivel de NSP4 fue E12, un marcador genético de RVA para Sudamérica, el cual además, fue observado en todas las 10 muestras para las cuales se estudió la constelación genética de RVA (Castells *et al*., 2020b).

Siguiendo con el estudio de la variabilidad genética, otro virus que mostró una gran diversidad fue BoNoV (Castells *et al*., 2020a). Se demostró la circulación de los dos genotipos en los cuales se encuentran agrupados actualmente, pero también se detectaron cepas de un posible tercer genotipo, cuarto del genogrupo GIII, siendo el principal genotipo detectado el GIII.2. Los resultados de las cepas que agrupan junto a cepas chinas recientemente descritas, además, apoyan la clasificación de estas cepas en un nuevo genotipo. Además, se detectaron 3 cepas recombinantes, las cuales agrupan conjuntamente con cepas recombinantes detectadas en diferentes regiones del mundo. Todas estas cepas recombinantes mencionadas, son GIII.P1-GIII.2, con punto de recombinación en la región de solapamiento ORF1-ORF2, lo cual indicaría que tienen un origen común, y se trata de una cepa recombinante que se dispersó mundialmente. Asimismo, una cuarta cepa recombinante fue detectada, la cual además, daría origen al nuevo genotipo mencionado

anteriormente (Castells *et al*., 2020a). Un análisis de coalescencia, permitió demostrar, en primer lugar, la presencia de 4 genotipos distintos, pero además, determinar que todas las cepas recombinantes GIII.P1-GIII.2 comparten un ancestro común, por lo que se confirma que se trató de un evento de recombinación dispersado mundialmente, y, de manera interesante, que el origen del genogrupo III posiblemente haya tenido lugar en nuestro país (Castells et al., 2021b).

La diversidad genética de BToV no fue ingente, probablemente en parte, debido a la baja frecuencia de detección. De todas maneras, dos linajes distintos fueron detectados. En el caso particular de BToV, todavía no se cuenta con una clasificación dentro de la especie, por lo cual las cepas detectadas no pudieron ser clasificadas más allá de pertenecer a BToV, por lo que sería interesante contar con un sistema de clasificación taxonómico dentro de la especie (Castells *et al*., 2021a).

En el caso de BoAstV, se observó una gran diversidad genética. Los BoAstV se clasifican dentro de 6 especies de *Mamastrovirus*, 4 de las cuales fueron detectadas, 3 de ellas en muestras provenientes de materia fecal, mientras que la restante en muestras de cerebro. Por otro lado, la gran mayoría de las cepas agruparon por fuera de todas las especies descritas hasta el momento, por lo que se siguieron todas las recomendaciones para confirmar que se trataba de una nueva especie, logrando efectivamente comprobarlo. En el caso de MAstV-13, especie que afecta al ganado vacuno a nivel del sistema nervioso central, se detectaron dos cepas, ambas relacionadas entre sí, obteniendo de una de ellas el genoma completo. Además, se logró determinar que el origen de esta especie se dio en Europa, llegando a nuestro país hace alrededor de 100 años, y se estimó la tasa de evolución en $4{,}27 \times 10^{-4}$ sustituciones por sitio por año. En el caso de MAstV-28, se detectó un único linaje, aunque interesantemente una de las cepas se detectó en una muestra de materia fecal pero de un ternero con síntomas respiratorios, y aparentemente MAstV-28 podría afectar a nivel del sistema respiratorio, por lo que nuestro resultado apoyaría esta teoría, aunque no es concluyente. Se detectó también la especie MAstV-33, también un único linaje. Por otro lado, se detectó una coinfección en un ternero, con la especie MAstV-29 y la nueva especie. Por último, de la especie clasificada en esta tesis, se detectaron 4 linajes, siendo además como se mencionó previamente, la más prevalente (Castells *et al*., 2019a; Giannitti *et al*., 2019, Doncel Díaz *et al*., 2021).

Por otro lado, no se observó la presencia ni de RVA ni de BCoV, en casos asociados al virus de la diarrea viral bovina. Para esto, se estudiaron 8 bovinos fallecidos naturalmente en 6 brotes espontáneos de enfermedades en establecimientos de ganado vacuno de carne y leche. En todos esos casos, se sospechó como causa principal de muerte el virus de la diarrea viral bovina, pero se estudiaron además otros posibles patógenos que pudieran estar afectando y empeorando el cuadro clínico. Sin embargo, como se mencionó al

principio, ni RVA ni BCoV se encontraron presentes en ninguno de los 8 bovinos analizados (da Silva *et al*., 2019). También se evaluó el efecto de 2 lactobacilos potencialmente probióticos sobre, entre otros factores, la abundancia de patógenos en las heces. Dentro de los patógenos estudiados, se incluyó RVA y BCoV. Sin embargo, a pesar de que se observó un beneficio en cuanto a la incidencia de diarrea, no se observó un claro efecto sobre la abundancia de patógenos, incluidos los virus mencionados (Fernandez *et al*., 2020). Además, se estudiaron las causas de diarrea neonatal de terneros y mortalidad en tambos a base de pastura en Uruguay, mediante un estudio de casos y controles. Se trató de un estudio genérico de la DNT, donde fueron incluidos, entre otros factores, los virus RVA, BCoV y BoAstV. Los resultados obtenidos concuerdan con lo observado para cada virus por separado, y se observó que la DNT es un síndrome multifactorial, donde RVA es de los patógenos más prevalentes y asociado a la DNT (Caffarena *et al*., 2021). Por último, se evaluó un producto basado en IgY para prevenir la diarrea por coronavirus bovino, con resultados prometedores donde se observó un retraso y reducción de la infección por BCoV y la diarrea, en terneros que recibieron el producto (Bok *et al*., 2020).

De todo lo anteriormente expuesto, se desprende que la situación de nuestro país, en cuanto a la presencia de virus entéricos bovinos en Uruguay es compleja, con detección tanto a nivel clínico pero también a nivel ambiental, y con una diversidad genética muy importante. La continuidad en la vigilancia de estos virus es fundamental para poder tomar medidas precisas con el fin de mejorar la salud animal y la producción ganadera en Uruguay.

6. Conclusiones

† Las metodologías moleculares desarrolladas en este trabajo fueron eficientes para la detección y caracterización de RVA, BCoV, BoNoV, BToV y BoAstV en muestras fecales de terneros y de RVA en aguas ambientales.

† RVA está frecuentemente presente en las aguas de los tambos, utilizadas para el consumo de los terneros, con un potencial de generar brotes de diarrea en los mismos.

† La frecuencia en la cual se detectaron los virus varió de 8% a 66%, demostrando una gran circulación de los mismos en terneros de Uruguay, principalmente de RVA y BoNoV.

† Los cinco virus estudiados: RVA, BoCoV, BoNoV, BoAstV y BoToV, fueron detectados en terneros provenientes de tambos en nuestro país.

† RVA, BoCoV, BoNoV y BoAstV fueron detectados en terneros de establecimientos dedicados a la producción de carne de nuestro país, mientras que BToV no fue detectado en este tipo de terneros.

† Se asoció a BoAstV con dos casos fatales de encefalitis, evidenciando el impacto de este virus más allá de las enteritis.

† Los terneros de hasta 3 semanas de vida se vieron mayormente afectados por los cinco virus estudiados.

† La vacunación demostró ser una herramienta efectiva para la prevención de BCoV, sin embargo, no lo fue para la prevención de RVA.

† Se observó una gran variabilidad genética en los cinco virus, advirtiendo sobre la necesidad de mantener la vigilancia epidemiológica molecular, para detectar emergencias de nuevas variantes genéticas con diferente potencial patogénico.

† Se describió una nueva especie de MAstV, con una gran circulación en Uruguay.

† Los virus estudiados en esta tesis evidencian una fuerte presencia en los terneros repercutiendo en su estado sanitario siendo responsables por una importante fracción de la DNT.

7. Perspectivas

† Continuar con la vigilancia y el análisis de los cinco virus estudiados en esta tesis. Este punto es sumamente importante, debido a que se observó una importante diversidad genética, lo cual puede tener implicancias directas en las medidas de control que se deban tomar.

† Extender el estudio a otros virus que también afectan a los bovinos a nivel entérico, entre los cuales se destacan nebovirus, enterovirus y kobuvirus. Conocer en detalle la diversidad de patógenos que circulan en nuestro país, así como también su frecuencia, es importante para complementarlo con el punto anterior, o sea, direccionar de forma más efectiva las estrategias para mejorar la producción bovina en nuestro país.

† Profundizar el estudio de los astrovirus que causan infecciones a nivel extra-intestinal, línea de investigación que surgió a raíz de la presente tesis. Así como fueron descritos dos casos de encefalitis asociada a BoAstV, se cuentan con datos preliminares que implicarían a BoAstV a nivel respiratorio.

† Aislar cepas locales de RVA y BCoV, que puedan utilizarse para la formulación de vacunas. Es importante, principalmente para RVA donde se demostró que las vacunas actuales no son efectivas, por lo que cepas locales podrían aumentar la eficacia. Para BCoV, contar con una metodología puesta a punto para aislar el virus es importante para, en caso de ser necesario, obtener cepas locales rápidamente para que puedan ser utilizadas en la formulación de la vacuna.

† Extender el estudio de virus entéricos a otras especies de hospederos, como los ovinos, también de gran importancia para nuestro país. Esto permitiría, además de conocer el grado de circulación de estos virus, conocer con mayor profundidad la dinámica de la transmisión interespecie. Está documentado, y los resultados obtenidos en esta tesis apoyan esta teoría, que la transmisión de virus entre ovinos y bovinos es frecuente.

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9. Artículos no relacionados a la tesis

Los trabajos que se mencionan a continuación no tienen vinculación con los objetivos de la tesis, sin embargo, fueron publicados durante el desarrollo de la misma, por lo que formaron parte del proceso de formación de doctorado.

Artículo 16. La historia evolutiva y dinámica espacio-temporal del linaje NC del virus de la tristeza de los cítricos

Benítez-Galeano MJ, **Castells M**, Colina R. The Evolutionary History and Spatiotemporal Dynamics of the NC Lineage of Citrus Tristeza Virus. Viruses. 2017 Oct 12;9(10). pii: E272. doi: 10.3390/v9100272.

De este trabajo soy co-primer autor.

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The Evolutionary History and Spatiotemporal Dynamics of the NC Lineage of Citrus Tristeza Virus

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Abstract: Citrus tristeza virus (CTV) is a major pathogen affecting citrus trees worldwide. However, few studies have focused on CTV's evolutionary history and geographic behavior. CTV is locally dispersed by an aphid vector and long distance dispersion due to transportation of contaminated material. With the aim to delve deeper into the CTV-NC (New Clade) genotype evolution, we estimated an evolution rate of 1.19×10^{-3} subs/site/year and the most common recent ancestor in 1977. Furthermore, the place of origin of the genotype was in the United States, and a great expansion of the population was observed in Uruguay. This expansion phase could be a consequence of the increment in the number of naïve citrus trees in Uruguayan orchards encompassing citrus industry growth in the past years.

Keywords: citrus tristeza virus; evolution rate; phylogeography; NC genotype

Artículo 17. Un análisis exhaustivo de la composición del genoma y los patrones de uso de codones de coronavirus emergentes

Tort FL, **Castells M**, Cristina J. A comprehensive analysis of genome composition and codon usage patterns of emerging coronaviruses. Virus Res. 2020;197976. doi:10.1016/j.virusres.2020.197976

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A comprehensive analysis of genome composition and codon usage patterns of emerging coronaviruses

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ABSTRACT

An outbreak of atypical pneumonia caused by a novel *Betacoronavirus* (βCoV), named SARS-CoV-2 has been declared a public health emergency of international concern by the World Health Organization. In order to gain insight into the emergence, evolution and adaptation of SARS-CoV-2 viruses, a comprehensive analysis of genome composition and codon usage of βCoV circulating in China was performed. A biased nucleotide composition was found for SARS-CoV-2 genome. This bias in genomic composition is reflected in its codon and amino acid usage patterns. The overall codon usage in SARS-CoV-2 is similar among themselves and slightly biased. Most of the highly frequent codons are A- and U-ending, which strongly suggests that mutational bias is the main force shaping codon usage in this virus. Significant differences in relative synonymous codon usage frequencies among SARS-CoV-2 and human cells were found. These differences are due to codon usage preferences.

Artículo 18. Evidencia de creciente diversificación de cepas emergentes de SARS-CoV-2

Castells M, Tort FL, Colina R, Cristina J. Evidence of increasing diversification of emerging SARS-CoV-2 strains. J Med Virol. 2020;1–8. https://doi.org/10.1002/jmv.26018

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RESEARCH ARTICLE

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Evidence of increasing diversification of emerging Severe Acute Respiratory Syndrome Coronavirus 2 strains

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Abstract

On 30th January 2020, an outbreak of atypical pneumonia caused by a novel betacoronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a public health emergency of international concern by the World Health Organization. For this reason, a detailed evolutionary analysis of SARS-CoV-2 strains currently circulating in different geographic regions of the world was performed. A compositional analysis as well as a Bayesian coalescent analysis of complete genome sequences of SARS-CoV-2 strains recently isolated in Europe, North America, South America, and Asia was performed. The results of these studies revealed a diversification of SARS-CoV-2 strains in three different genetic clades. Co-circulation of different clades in different countries, as well as different genetic lineages within different clades were observed. The time of the most recent common ancestor was established to be around 1st November 2019. A mean rate of evolution of 6.57×10^{-4} substitutions per site per year was found. A significant migration rate per genetic lineage per year from Europe to South America was also observed. The results of these studies revealed an increasing diversification of SARS-CoV-2 strains. High evolutionary rates and fast population growth characterizes the population dynamics of SARS-CoV-2 strains.

KEYWORDS
coalescent, coronavirus, evolution, SARS-CoV-2

Artículo 19. Detección molecular de norovirus en muestras de aguas residuales uruguayas revela una alta diversidad genética y el reemplazo de variantes GII.4 a lo largo del tiempo

Victoria M, Tort LF, Lizasoain A, García M, **Castells M**, Berois M, Divizia M, Leite JP, Miagostovich MP, Cristina J, Colina R. Norovirus molecular detection in Uruguayan sewage samples reveals a high genetic diversity and GII.4 variant replacement along time. J Appl Microbiol. 2016 May;120(5):1427-35. doi:10.1111/jam.13058.

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ORIGINAL ARTICLE

Norovirus molecular detection in Uruguayan sewage samples reveals a high genetic diversity and GII.4 variant replacement along time

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Keywords
genetic diversity, genotypes, norovirus, sewage, variant replacement.

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Abstract

Aims: To determine the prevalence and molecular epidemiology of norovirus (NoV) genogroup I (GI) and GII in Uruguay.
Methods and Results: One hundred and sixteen sewage samples were collected in six cities (Bella Unión, Salto, Paysandú, Fray Bentos, Melo and Treinta y Tres) from March 2011 to April 2013, viruses were concentrated by ultracentrifugation and NoV studies were performed by semi-nested RT-PCR (partial capsid region). NoV were detected in samples from all the cities and detected in 72% (84/116) of the samples with nine of them belonging to GI, 48 to GII and 27 to both genogroups. Remarkably, a high genetic diversity was identified: GII.2 ($n = 13$), GII.4 ($n = 13$), GI.1 ($n = 5$), GI.4 ($n = 5$), GI.8 ($n = 4$), GII.13 ($n = 4$), GII.1 ($n = 3$), GII.6 ($n = 3$), GI.3 ($n = 1$), GI.5 ($n = 1$), GI.6 ($n = 1$), GII.3 ($n = 1$), GII.17 ($n = 1$). Interestingly, a complete replacement of GII.4 New Orleans 2009 by GII.4 Sydney 2012 variants during 2012 was evidenced.
Conclusion: This study reveals a high circulation of different NoV GI and GII genotypes in sewage evidencing a replacement of GII.4 variants.
Significance and Impact of Study: This approach can be used as an indicator of the presence of a new GII.4 variant which can originate an increase in acute gastroenteritis outbreaks worldwide.

Artículo 20. Bocavirus humano: detección, cuantificación y caracterización molecular en aguas residuales y superficiales en Uruguay

Salvo M, Lizasoain A, **Castells M**, Bortagaray V, Castro S, Colina R, Tort FL, Victoria M. Human Bocavirus: Detection, Quantification and Molecular Characterization in Sewage and Surface Waters in Uruguay. Food Environ Virol. 2018 Jun;10(2):193-200. doi: 10.1007/s12560-017-9334-0.

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ORIGINAL PAPER

Human Bocavirus: Detection, Quantification and Molecular Characterization in Sewage and Surface Waters in Uruguay

Matías Salvo[1] · Andrés Lizasoain[1] · Matías Castells[1] · Viviana Bortagaray[1] · Sebastián Castro[2] · Rodney Colina[1] · Fernando Lopez Tort[1] · Matías Victoria[1]

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Abstract
Human bocavirus (HBoV) infections are related to respiratory and gastroenteric diseases. The aim of this study was to investigate the presence of HBoV in both sewage and surface waters in Uruguay. Sixty-eight sewage samples from the cities of Salto, Paysandú, Bella Unión, Fray Bentos, Treinta y Tres and Melo and 36 surface water samples from the cities of Salto, Florida and Santa Lucía were studied. HBoV was screened by multiplex qPCR for the detection of the four subtypes, followed by monoplex qPCRs for the independent quantification of each subtype. A qualitative PCR followed by DNA sequencing and phylogenetic analysis was carried out for molecular characterization of HBoV strains. HBoV was present in a high frequency (69%) in sewage and only one positive sample (3%) was found in surface water. Concerning sewage samples, HBoV1 was detected in 11 (23%) out of the 47 positives samples, with a mean concentration of 8.2×10^4 genomic copies/Liter (gc/L), HBoV3 was detected in 35 (74%) of the positive samples with a mean concentration of 4.1×10^6 gc/L and subtypes 2 and/or 4 were detected in 39 (83%) of the positive samples with a mean concentration of 7.8×10^6 gc/L. After the phylogenetic analysis performed by a Bayesian approach, the four HBoV subtypes were confirmed. This is the first study determining a high frequency of HBoV and the presence of the four HBoV subtypes in aquatic matrices in Latin America, mainly in sewage. Although HBoV was scarcely detected in surface water, a waterborne transmission is likely to occur if people enter in contact with polluted surface waters for recreational activities such as fishing or swimming since an elevated frequency of HBoV was detected in raw sewage which is usually directly discharged into surface waters.

Keywords Human bocavirus · Sewage · Surface water · qPCR · Molecular characterization · Uruguay

Artículo 21. Un extenso estudio de campo revela la circulación de nuevas variantes genéticas del subtipo 1a de BVDV en Uruguay

Maya L, Macías-Rioseco M, Silveira C, Giannitti F, **Castells M**, Salvo M, Rivero R, Cristina J, Gianneechini E, Puentes R, Flores EF, Riet-Correa F, Colina R. An extensive field study reveals the circulation of new genetic variants of subtype 1a of bovine viral diarrhea virus in Uruguay. Arch Virol. 2020 Jan;165(1):145-156. doi: 10.1007/s00705-019-04446-z.

Archives of Virology
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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.

Artículo 22. Evaluación de la contaminación bacteriana como indicador de contaminación viral en un acuífero sedimentario en Uruguay

Gamazo P, Victoria M, Schijven JF, Alvareda E, Tort LFL, Ramos J, Burutaran L, Olivera M, Lizasoain A, Sapriza G, **Castells M**, Colina R. Evaluation of Bacterial Contamination as an Indicator of Viral Contamination in a Sedimentary Aquifer in Uruguay. Food Environ Virol. 2018 Sep;10(3):305-315. doi: 10.1007/s12560-018-9341-9.

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ORIGINAL PAPER

Evaluation of Bacterial Contamination as an Indicator of Viral Contamination in a Sedimentary Aquifer in Uruguay

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Abstract
In Uruguay, groundwater is frequently used for agricultural activities, as well as for human consumption in urban and rural areas. As in many countries worldwide, drinking water microbiological quality is evaluated only according to bacteriological standards and virological analyses are not mentioned in the legislation. In this work, the incidence of human viral (Rotavirus A, Norovirus GII, and human Adenovirus) and bacterial (total and thermotolerant coliform and *Pseudomonas aeruginosa*) contamination in groundwater in the Salto district, Uruguay, as well as the possible correlation between these groups of microorganisms, was studied. From a total of 134 groundwater samples, 42 (32.1%) were positive for Rotavirus, only 1 (0.7%) for both Rotavirus and Adenovirus, and 96 (72.6%) samples were positive for bacterial indicators. Results also show that Rotavirus presence was not associated with changes in chemical composition of the aquifer water. Bacteriological indicators were not adequate to predict the presence of viruses in individual groundwater samples (well scale), but a deeper spatial–temporal analysis showed that they are promising candidates to assess the viral contamination degree at aquifer scale, since from the number of wells with bacterial contamination the number of wells with viral contamination could be estimated.

Artículo 23. Modelado del transporte de rotavirus y norovirus humanos en sistemas de matrices acuáticas estandarizados y en suelo natural

Gamazo P, Victoria M, Schijven JF, Alvareda E, Tort LFL, Ramos J, Lizasoain LA, Sapriza G, **Castells M**, Bessone L, Colina R. Modeling the Transport of Human Rotavirus and Norovirus in Standardized and in Natural Soil Matrix-Water Systems. Food Environ Virol. 2020 Mar;12(1):58-67. doi: 10.1007/s12560-019-09414-z.

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ORIGINAL PAPER

Modeling the Transport of Human Rotavirus and Norovirus in Standardized and in Natural Soil Matrix-Water Systems

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Abstract
We modeled Group A Rotavirus (RVA) and Norovirus genogroup II (GII NoV) transport experiments in standardized (crystal quartz sand and deionized water with adjusted pH and ionic strength) and natural soil matrix-water systems (MWS). On the one hand, in the standardized MWS, Rotavirus and Norovirus showed very similar breakthrough curves (BTCs), showing a removal rate of 2 and 1.7 $\log_{10}$, respectively. From the numerical modeling of the experiment, transport parameters of the same order of magnitude were obtained for both viruses. On the other hand, in the natural MWS, the two viruses show very different BTCs. The Norovirus transport model showed significant changes; BTC showed a removal rate of 4 $\log_{10}$, while Rotavirus showed a removal rate of 2.6 $\log_{10}$ similar to the 2 $\log_{10}$ observed on the standardized MWS. One possible explanation for this differential behavior is the difference in the isoelectric point value of these two viruses and the increase of the ionic strength on the natural MWS.

10. Consideraciones finales

Esta tesis permitió ahondar y en algunos casos estudiar por primera vez en Uruguay, virus que afectan la salud animal, particularmente a los bovinos, con un gran impacto negativo en la producción ganadera, conllevando un impacto negativo también en la economía de nuestro país.

Durante el desarrollo de la tesis, publiqué 20 artículos científicos en revistas arbitradas internacionales, en los cuales tuve participación activa, siendo pilares fundamentales en lo que fue mi formación a nivel de doctorado. Además de los 20 artículos publicados, hay otros 3 que se encuentran en proceso de publicación. Si bien algunos de estos trabajos no está vinculado directamente a esta tesis, sí lo estuvieron en lo que a mi formación respecta.

Los resultados obtenidos en esta tesis, dieron origen a 15 artículos científicos. A su vez, de los 15 artículos, 10 de ellos están directamente relacionados, siendo artículos principales de la presente tesis. Al momento de la presentación de esta tesis, 8 de los 10 artículos principales se encuentran publicados en revistas arbitradas internacionales. En 9 artículos soy el primer autor, mientras que en el restante soy el último autor y de correspondencia; en 6 además de primer autor, soy co-autor de correspondencia (4 de ellos publicados, y 2 en proceso de publicación). Los resultados obtenidos, a su vez, darán origen a nuevos estudios, dado que hay perspectivas de continuar y ahondar en el estudio evolutivo de los virus entéricos bovinos.

Por otro lado, además de participar en diversos proyectos de investigación, fui responsable de uno de ellos, obteniendo resultados de gran importancia para el desarrollo y resultados de esta tesis.

Por útimo, destacar la invitación de 16 revistas internacionales arbitradas, para ser revisor de manuscritos enviados a las mismas. Las revistas para las cuales revisé artículos durante el desarrollo de esta tesis fueron: Archives of Virology; Virus Research; Journal of Genetics; BMC Veterinary Research; Coronaviruses; Journal of Virological Methods; Beneficial Microbes; Biochemical Genetics; Frontiers in Microbiology; Gene Reports; Genomics; Infection, Genetics and Evolution; Journal of Molecular Evolution; Revista do Instituto de Medicina Tropical de São Paulo; Veterinary Medicine and Science; VirusDisease. En total, fui invitado a revisar más de 30 artículos científicos durante el desarrollo de esta tesis. Además, fui evaluador de un proyecto ANII. Este proceso fue también importante para mi formación como investigador a nivel de doctorado.