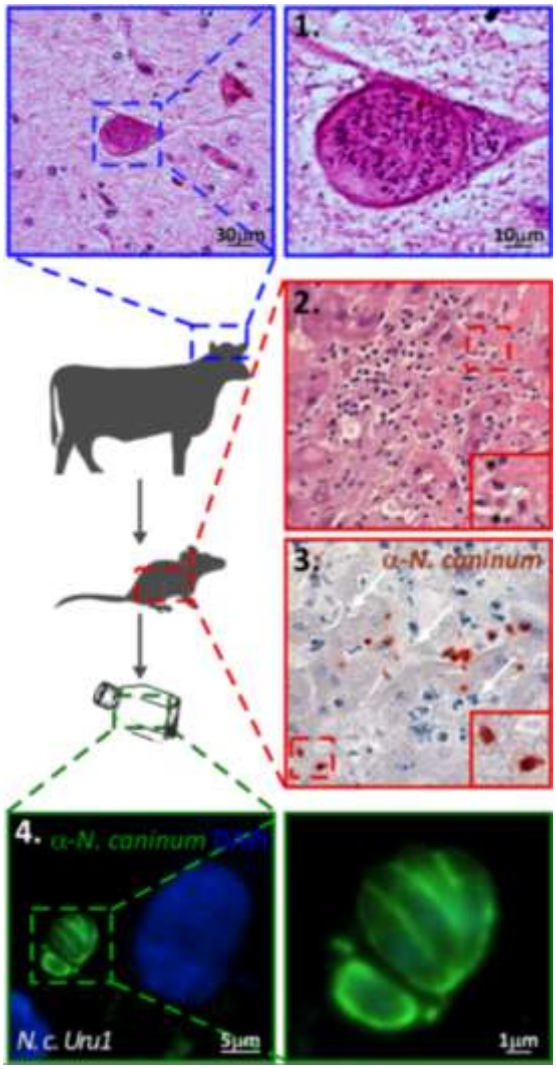
Tesis de doctorado en Ciencias biológicas.

PEDECIBA

“Bases moleculares interacción hospedero-pátogeno en neosporosis bovina”

Andrés Cabrera



Orientador: Dr. Carlos Robello

Co-Orientadores: Dra. María Eugenia Francia

Dr. Otto Pritsch

Comité evaluador:

Dra. Leticia Zarantonelli

Dra. Leticia Pérez

Dr. Rodrigo Puentes

Institut Pasteur Montevideo.

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1.0 Introducción a la neosporosis bovina

1.1 Importancia del tema

La neosporosis bovina es una enfermedad causada por el parásito *Neospora caninum* y afecta principalmente al ganado bovino, presentando una amplia distribución mundial (Anderson et al., 2000; Dubey, 2005). Las pérdidas económicas por este parásito fueron cuantificadas en varios países y en diferentes contextos productivos (Tabla 1), por lo que es conocida como la enfermedad del billón de dólares (Reichel et al., 2013). Por ejemplo, las pérdidas económicas cuantificadas en países europeos como España, Reino Unido y Holanda describen entre 9 a 30 millones de dólares (Reichel et al., 2013), mientras que en Estados Unidos se calculan pérdidas anuales de 546 millones de dólares en la industria lechera y 110 millones dólares en la industria cárnica. La región no es ajena a este problema: en Argentina por ejemplo, se calcularon pérdidas anuales relacionadas con esta enfermedad por un monto total de 56 millones de dólares (Moore et al., 2013).

Tabla 1. Cuantificación de las pérdidas económicas producidas por *N. caninu*m. Adaptado de Reichel et al., 2013.

País	Industria	Perdidas economicas totales (millonesde dólares)
Argentina	Lechería	38.5
	Cárnica	48.9
Australia	Lechería	26.6
	Cárnica	74.1
Brasil	Lechería	51.3
	Cárnica	101.0
Canadá	Lechería	17.1
	Cárnica	14.3
España	Lechería	19.8
	Cárnica	9.8
Estados Unidos	Lechería	546.3
	Cárnica	111.4
Holanda	Lechería	12.1
Mexico	Lechería	68.5
	Cárnica	94.8
Nueva Zelanda	Lechería	35.7
	Cárnica	1.1
Reino Unido	Lechería	27.0

En los últimos 16 años, Uruguay ha tenido un proceso de crecimiento económico ininterrumpido, cuadruplicando el ingreso per cápita (medido en dólares corrientes), que alcanzó US$17.232 en 2018 (Fuente: Uruguay XXI en base a BCU y Cepal). El sector agropecuario y las cadenas agroindustriales tienen un rol clave en la economía y fueron los responsables de dicho dinamismo, explicando aproximadamente el 11% del PIB uruguayo en 2018. Dicha participación se repartió entre el sector primario (agricultura, ganadería y silvicultura) y las industrias relacionadas con el agro, con 6% y 5% respectivamente. Por otra parte, la industria agropecuaria ocupa en forma directa a 150.000 personas, lo que representa casi el 10% del total del personal ocupado en el país. Dentro de este sector, la mayoría se dedica a la cría (60.586) seguido por la explotación agropecuaria mixta (24.454). Con un mercado interno reducido, la producción del sector agroindustrial del país se destina mayoritariamente a la exportación. En este sentido, las exportaciones totales de alimentos cubren el equivalente a las necesidades alimenticias de 28

millones de personas. El 32% de las exportaciones uruguayas de bienes y servicios del último año, unos 2.900 millones de dólares, provinieron del sector ganadero y lechero, siendo la carne bovina el principal rubro exportable del país (20% del total), con los productos lácteos en el cuarto lugar (7%), detrás de la celulosa y la soja. Estas cifras no hacen más que destacar la importancia de las cadenas cárnicas y lechera en la economía de nuestro país y determinan que cualquier actividad que afecte a los niveles de productividad y reproductividad de los rodeos tendrá un alto impacto en la misma (https://www.gub.uy/ministerio-economia-finanzas).

1.2 Antecedentes históricos

El parásito *Neospora caninum* fue descubierto en el año 1984 por Bjerkas y colaboradores y se lo se lo vinculó inicialmente a perros que presentaban sintomatología nerviosa grave caracterizada por meningoencefalomielitis y miositis. Fue confundido con el parásito *Toxoplasma gondii* por formar el mismo tipo de quiste y presentar la misma estructura visualizada al microscopio óptico (Bjerkås et al., 1984). Posteriormente se identificó el mismo agente asociado a cuadros de encefalomielitis en terneros neonatos (Parish et al., 1987). Es en 1988 cuando Dubey y colaboradores proponen describir la nueva especie como *Neospora caninum*, generando un nuevo género *Neospora* tras identificar el parásito en perros que presentaban la misma sintomatología clínica descrita por Bjerkas. Es así que el primer aislamiento de este nuevo parásito se obtuvo del tejido nervioso de estos perros con sintomatología, utilizando el modelo ratón para su aislamiento. Cabe destacar que se realizó la infección experimental de perros sanos logrando producir enfermedad en los mismos.

No fue hasta 1989 en una granja de Nuevo México (USA) que se relacionó la neosporosis como un agente que producía aborto en el ganado bovino (Thilsted and Dubey, 1989). Posteriormente la infección por este parásito se asoció a las pérdidas reproductivas ocasionadas en el ganado bovino en varios países (Dubey and Lindsay, 1996). En 1992 se comprueba experimentalmente la transmisión vertical del parásito en la especie bovina (Dubey et al., 1992), y un año después se obtiene el primer aislado de *N. caninum* a partir de un feto bovino (Conrad et al., 1993). Desde entonces, se han realizado numerosos estudios de prevalencia de la infección en diversas regiones del mundo, tanto en animales adultos como en fetos abortados, pasando la neosporosis a considerarse como una de las principales causas de aborto bovino a nivel mundial (Dubey et al., 2007a).

El descubrimiento reciente de este parásito y su impacto en las ciencias veterinarias generaron un amplio interés de las investigaciones científicas que va desde la enfermedad y su impacto, la biología del parásito y de la interacción hospedero patógeno, así como la epidemiología de la enfermedad y su profilaxis.

1.3 Taxonomía del agente

N. caninum es un parásito coccidio intracelular obligatorio perteneciente al phylum Apicomplexa. Este phylum se caracteriza por la disposición típica de algunas de sus organelos (conoide, roptrias y anillo polar) en la parte anterior de la célula, formando lo que se denomina complejo apical. Además, se incluye dentro de la familia *Sarcocystidae*, que comprende otros géneros de apicomplejos de ciclo biológico heteroxeno y formadores de quistes tisulares (*Toxoplasma, Hammondia, Besnoitia, Sarcocystis y Frenkelia*). Las diferencias proteicas,

antigénicas, estructurales, moleculares y biológicas entre *Neospora* y los otros integrantes de la familia *Sarcocystidae* son suficientemente significativas como para considerarlos géneros diferentes (Dubey et al., 2002).

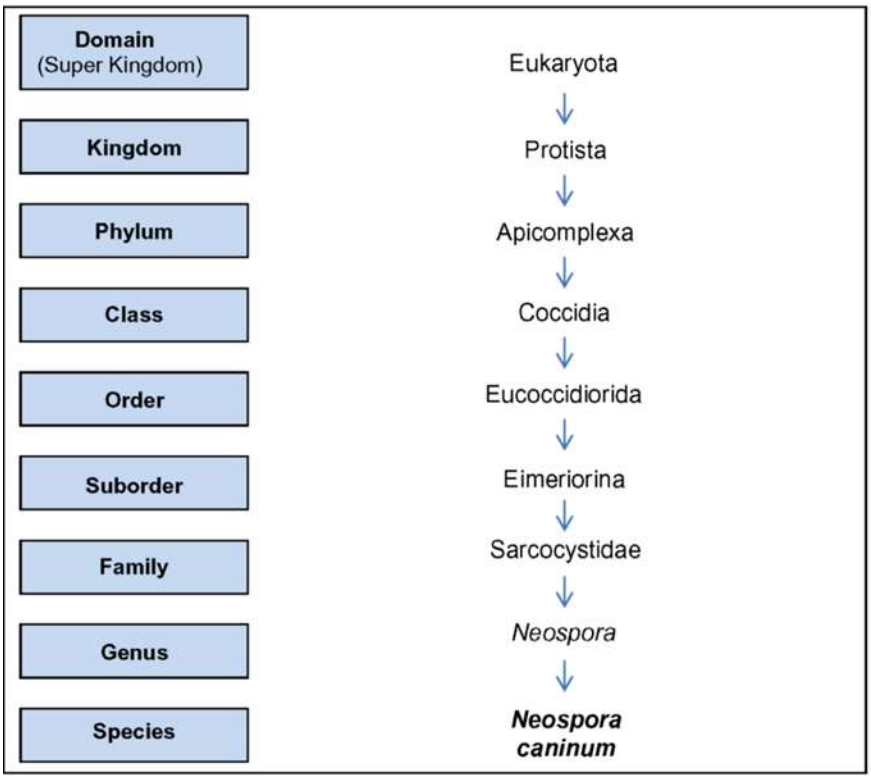

Figure 1. Clasificación taxonómica de N. caninum (Goodswen et al., 2013).

El género Neospora comprende la especie *Neospora caninum* y *Neospora hughesi*, esta última aislada del tejido nervioso de un caballo adulto con sintomatología nerviosa (Marsh et al., 1998, 1999). En la figura 1 se muestra la clasificación taxonómica del parásito.

1.4 Estadios del parásito y su ciclo biológico

El parásito *N. caninum* presenta tres estadios bien diferenciados (Figura 2): taquizoitos (forma replicativa), bradizoitos (forma resistente) y esporozoitos en el interior de ooquistes (forma ambiental. Los taquizoitos y los quistes tisulares son los estadíos infectantes que se localizan en los diversos tejidos del hospedador intermediario, mientras que los ooquistes son eliminados al medio ambiente con las heces del hospedador definitivo (cánidos).

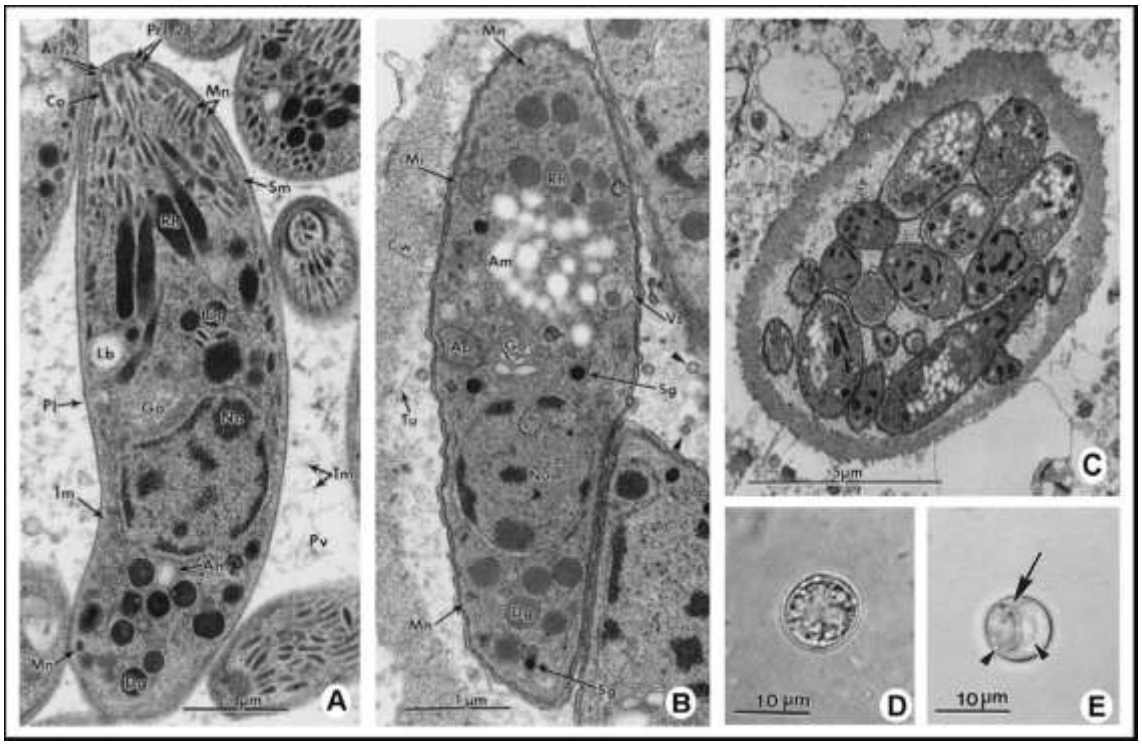

Figure 2. Microscopia electrónica de los diferentes estadios de N. caninum. A. Taquizoito, B. Bradizoito, C. Quiste tisular, D. Ooquiste esporulado, E. Ooquiste con esporozoitos. (Co) Conoide, (Ap) Apicoplasto, (Mn) Micronemas, (Rh) Roptrias, (Dg) Gránulos densos, (PV) Vesícula parasitófora (Goodswen et al., 2013).

Los taquizoitos (tachos= rápidos en griego) son formas invasivas y se caracterizan por la rápida replicación. Este estadio es el causante de la fase aguda de la infección y el encargado de la diseminación del parásito a los diferentes órganos blancos causando el daño tisular, responsable

de la clínica de la enfermedad, en el hospedador intermediario (Dubey and Lindsay, 2006). Poseen un tamaño que varía entre 3 y 7 μm y su morfología puede ser ovoide, lunar u ovalada, en función de la etapa de división en la que se encuentren (Dubey et al., 2002).

Como se puede observar en la figura 2, el taquizoíto presenta todas las estructuras características de los parásitos apicomplejos. En la misma se reconoce su membrana, que está compuesta por la membrana plasmática y el complejo interno de membrana, formado a su vez por una serie de alveolos aplanados que interaccionan externamente con el glideosoma e internamente con los mricrotúbulos subpeliculares (Ouologuem and Roos, 2014). El glideosoma actúa como el sistema motor y las principales proteínas que lo constituyen son la actina, la miosina y un grupo de enzimas denominadas "GAP" del inglés glideosome-associated proteins (Jacot et al., 2014). A su vez los microtúbulos subpeliculares recorren longitudinalmente el citoplasma y ejercen la función de citoesqueleto, uniéndose en dos anillos polares anteriores, entre los que se encuentra el conoide, y extendiéndose hasta el segundo tercio de la célula en el extremo posterior (Speer and Dubey, 1989). El conoide se define como un cono truncado y móvil de filamentos de tubulina modificada con un anillo polar posterior (Lindsay et al., 1993). Este elemento del citoesqueleto se encuentra conservado únicamente en las especies conocidas como coccidias dentro de los Apicomplejos; aquellas cuyo ciclo de vida involucra una fase entérica. También se observan tres organelos características del filo: las roptrias, los micronemas y los gránulos densos, generalmente las dos primeras distribuidas principalmente en la porción apical del parásito y la ultima en todo el citoplasma (Speer et al., 1999). Las proteínas secretadas roptrias, muchas de ellas quinasas, cumplen la función de ayudar a la formación y maduración de la vacuola parasitófora dentro de la célula infectada y constituyen uno de los factores de virulencia (Beck et al., 2014). En este sentido se describieron varios genes que codifican para diferentes proteínas de las roptrias en *N. caninum*

(Talevich and Kannan, 2013). Como ejemplo de ellos una de las más importantes es NcROP40, donde se observa la expresión diferencial en las cepas de mayor virulencia (Pastor-Fernández et al., 2015). Los micronemas son pequeñas vesículas que secretan proteínas al exterior, participando en el reconocimiento y adhesión a la célula hospedadora (Hemphill et al., 2013). Cabe destacar que al conjunto de los organelos conoide, anillos polares, micronemas y roptrias se los conoce como complejo apical (Speer and Dubey, 1989). Los gránulos densos participan activamente de la secreción de productos para formar y mantener la vacuola parasitófora en el interior de la célula hospedadora (Hemphill, 1999), y a su vez participan en la modulación de la respuesta inmune de la célula infectada a través de la manipulación de su expresión génica, siendo conjuntamente con las roptrias importantes factores de virulencia (Ma et al., 2017b, 2017a). Estudios de proteína gránulo densa 7 (GRA7) constataron la capacidad de ésta de regular la patogénesis mediante el control de la respuesta inmune del hospedero (Yoshifumi et al., 2018).

Los bradizoítos son el estadio que genera la infección crónica de la enfermedad en el hospedero intermediario. Se caracterizan por su multiplicación lenta y generar los quistes tisulares en el hospedero. Su tamaño varía aproximadamente 5-8 µm de largo y 1-2 µm de ancho y poseen las mismas estructuras que el taquizoíto, aunque existen ciertas diferencias en cuanto a la disposición y número de las organelos secretores en su interior (Speer & Dubey, 1989; Dubey et al., 2004). Particularmente este estadio se encuentra en quistes tisulares en el tejido del sistema nervioso central y principalmente en tejido muscular esquelético (Dubey and Lindsay, 1996). Los quistes tisulares tienen una forma redondeada u oval y generalmente miden unos 100µm de diámetro. Están delimitados por una membrana quística de hasta 4µm de espesor (Peters et al., 2001). Dentro de los mismos pueden encontrarse hasta 200 bradizoitos (Dubey, 2003).

Por último los esporozoitos se localizan en el interior de los ooquistes, siendo estos últimos las formas de resistencia ambiental del parásito que son eliminados con las heces del hospedero definitivo (Dubey, 2003). Los ooquistes se eliminan al medio sin esporular y deben sufrir una ronda de esporogonia para ser infectivos (McAllister et al., 1998). La esporulación se completa, en condiciones adecuadas de temperatura y humedad, transcurridas aproximadamente 24h. El ooquiste esporulado, morfológicamente muy similar a los de Toxoplasma y Hammondia, tiene forma esférica y su tamaño es aproximadamente de 12 μm de longitud y 11 μm de ancho (King et al., 2010). En su interior se encuentran dos esporoquistes que a su vez contienen cuatro esporozoítos (Dubey et al., 2002).

El ciclo biológico del parásito se clasifica como heteroxeno facultativo, es decir tienen dos tipos de hospedadores: uno definitivo (cánidos) y otro intermediario (herbívoros en su gran mayoría) (McAllister et al., 1998). Cabe destacar que las fases de multiplicación sexual del parásito se desarrollan en los hospederos definitivos, en cambio la multiplicación asexual del mismo se registra en los hospedadores intermediarios (Figura 3) (Dubey, 2003).

Hasta la fecha se han descrito como hospedadores definitivos, el perro (McAllister et al., 1998), el coyote (Gondim et al., 2004), el dingo (King et al., 2010) y el lobo (Dubey et al., 2011). Por su parte, como hospedadores intermediarios intervienen, principalmente, el ganado bovino y ovino (Thilsted and Dubey, 1989), siendo el hospedador intermediario más frecuente el primero. La presencia de *N. caninum* en pequeños rumiantes está cobrando cada vez más importancia como agente abortivo (Moreno et al., 2012; Unzaga et al., 2014). La infección por *N. caninum* ha sido detectado por diversas técnicas diagnósticas en un amplio espectro de animales domésticos y silvestres, como por ejemplo en cerdos, roedores, aves entre otros (Mineo et al., 2009; Moré et al., 2008; Muradian et al., 2012; Nazari et al., 2020; Snak et al., 2019). Por otro lado, son pocas, pero

no inexistentes, las evidencias de que *N. caninum* pueda infectar al ser humano (Duarte et al., 2020; Ibrahim et al., 2009; Oshiro et al., 2015).

El ciclo comienza con la eliminación de ooquistes al ambiente por los diferentes hospedadores definitivos (Dubey et al., 2007a, 2011; McAllister et al., 1998). La formación de estos ooquistes se dan en las células enteroepiteliales de los diferentes hospedadores definitivos (Kul et al., 2015). Una vez en el ambiente, los ooquistes deben sufrir una esporulación para resultar infectantes. Al igual que en otros coccidios, aparentemente los ooquistes tienen una resistencia ambiental muy elevada (Alves Neto et al., 2011).

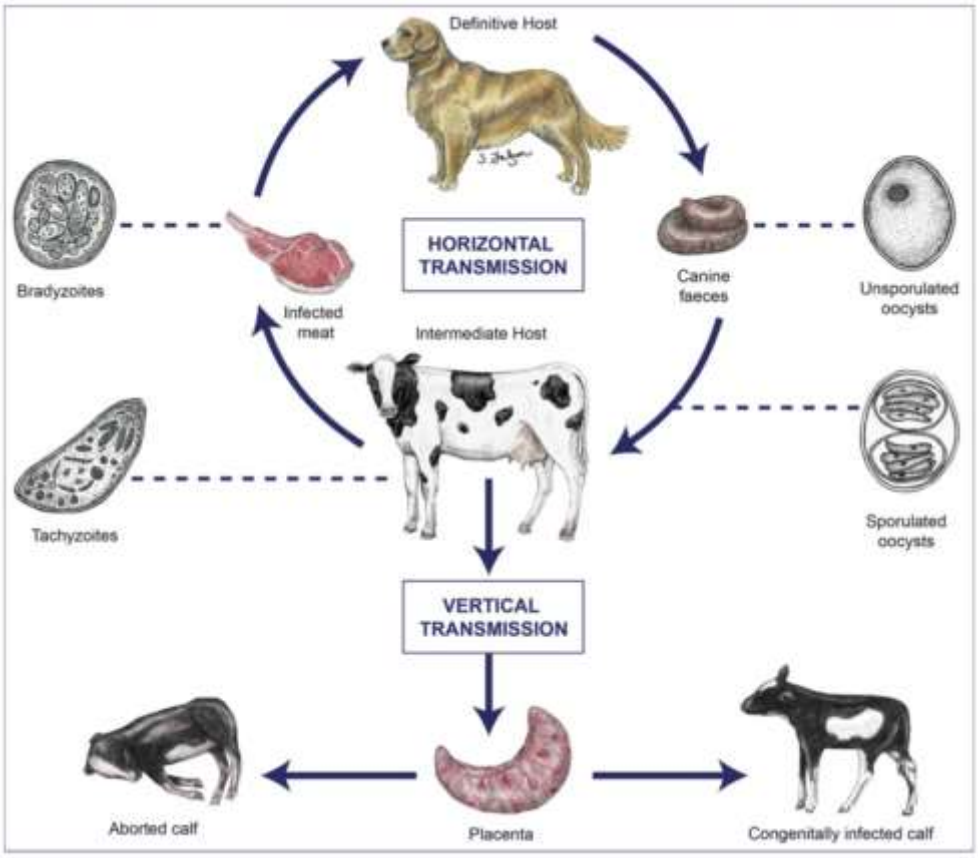

Figura 3. Ciclo biológico de N. caninum (Goodswen et al., 2013)

La ingestión por parte de hospederos intermediarios, de alimentos o agua contaminados con ooquistes esporulados da continuidad al ciclo (Dubey, 2003). En el intestino de los hospederos intermediarios, se liberan los esporozoítos que infectan las células del epitelio intestinal (Dubey, 2003). Ahí ocurre la transformación al estadio taquizoíto, donde se multiplican masivamente. Posteriormente, alcanzan el torrente circulatorio a través de los linfonódulos mesentéricos, produciéndose la diseminación intraorgánica del parásito. Los taquizoítos pueden invadir una amplia variedad de órganos y tejidos tales como el corazón, pulmón, hígado, músculo esquelético, placenta, cerebro o piel (Dubey, 2003; Dubey and Lindsay, 1996). Con el tiempo, el hospedador desarrolla una respuesta inmunitaria capaz de eliminar la mayor parte de los taquizoítos, si bien algunos de ellos logran evadir dicha respuesta mediante la conversión al estado latente de bradizoíto, secuestrándose principalmente en el sistema nervioso central (SNC) y tejido muscular esquelético, iniciando así la fase crónica de la infección (Buxton et al., 2002; Peters et al., 2001). De esta forma, el parásito permanece en el hospedador durante periodos prolongados de tiempo sin mostrar signos clínicos (Dubey et al., 2006).

La depredación de tejidos infectados con quistes de bradizoítos por el hospedador definitivo es la única vía de transmisión del hospedador intermediario infectado, cerrando de esta forma el ciclo biológico (Dubey and Schares, 2011). Además, en el caso del hospedador intermediario debe tenerse en cuenta que también se produce la infección de forma vertical por la vía transplacentaria (Dubey, 2003). De importancia para el sector productivo de nuestro país, cabe destacar que, se ha podido establecer la existencia de un ciclo silvestre además del ciclo doméstico ya descrito (Gondim, 2006). El parásito es capaz de circular entre ambos, siendo posible, por tanto, la transmisión del parásito de animales silvestres al perro y, posteriormente, de éste al ganado

bovino, así como del ganado bovino a cánidos silvestres, y de éstos a los rumiantes silvestres (Gondim et al., 2004).

1.5 Aislados y su variabilidad genética

Desde su descubrimiento hace unos 40 años, los aislamientos de *N. caninum* han incrementado significativamente (Al-Qassab et al., 2010a). Sin embargo, el número de aislamientos es aún escaso, considerando las pérdidas económicas que causa el parásito (Dubey and Lindsay, 1996). Su reciente descubrimiento, la poca información sobre su biología y la dificultad técnica para realizar los aislamientos explicarían las pocas cepas aisladas de este patógeno (Al-Qassab et al., 2010a; Dubey et al., 2007a; Rojo-Montejo et al., 2009b). El primer aislamiento canino (NC1) fue obtenido en 1988 por Dubey y los primeros aislamientos bovinos (BPA1 y BPA2) fueron realizados por Conrad, ambos de los Estados Unidos (Conrad et al., 1993; Dubey et al., 1988). Si bien inicialmente la mayoría de los aislamientos fueron de origen canino y bovino, en los últimos tiempos se ha logrado la obtención de nuevos aislamientos de una gran diversidad de animales (Al-Qassab et al., 2010a; Schock et al., 2001).

Esa diversidad comprende desde animales domésticos como la vaca, oveja, perro y caballo a silvestres como ser ciervo, búfalo, bisonte y lobo (Dubey et al., 2011; Dubey and Schares, 2011). A la diversidad de origen animal, se le suma la diversidad de orígenes geográficos de las diferentes cepas (Al-Qassab et al., 2010a). Si bien la mayoría de los aislamientos que se generaron en estos

30 años son de origen europeo, donde están representados mayoritariamente por España, Reino Unido, Alemania, Portugal, Polonia, República Checa, Suécia e Italia (Barber et al., 1993; Goździk and Cabaj, 2007; Regidor-Cerrillo et al., 2020; Rojo-Montejo et al., 2009a; Šlapeta et al., 2002; Stenlund et al., 1997), se han reportado también varios aislamientos simultáneamente en Norteamérica (Cuddon et al., 1992; Dubey et al., 1992, 2007b). Asimismo, se le suman aislamientos de diferentes partes del sudeste asiático y oriente medio, donde se destacan países como Japón, Corea del Sur, China, Malasia e Irán (Cheah et al., 2004; Jia et al., 2014; Kim et al., 2000; Salehi et al., 2012; Yamane et al., 1997). Australia y Nueva Zelanda también generaron sus propios aislamientos, representando así a Oceanía (Miller et al., 2002; Okeoma et al., 2004). En Sudamérica, al comienzo de esta tesis doctoral, únicamente en Brasil y Argentina se obtuvieron aislamientos autóctonos (Basso et al., 2001; García-Melo et al., 2009; Gondim et al., 2001; Pena et al., 2007).

La diversidad de aislados de diferentes orígenes animales y geográficos, y la variabilidad intra-específica de los fenotipos generó la necesidad de caracterizar genéticamente las cepas (Al-Qassab et al., 2009). En este sentido, los primeros estudios de variabilidad genética se basaron en el análisis del ARNr 18S así como de la región ITS1, y mediante el uso de la técnica de los RAPD. Debido a la elevada conservación de las secuencias de los marcadores analizados, estos abordajes indicaban una menor variabilidad genética que la descrita para *T. gondii* (Beck et al., 2009). Posteriormente, el análisis de secuencias de microsatélites permitió evidenciar una elevada diversidad genética entre los aislados del parásito obtenidos in vitro (Regidor-Cerrillo et al., 2006) y a partir de muestras de animales con infección natural (Basso et al., 2010). Gracias al análisis multilocus, ha sido posible definir una estructuración parcial de *N. caninum* en la que ciertos perfiles genéticos de cada aislado se han asociado a su origen geográfico (Regidor-Cerrillo et al.,

2013). Si bien los microsatélites permiten caracterizar genéticamente al parásito, estos no necesariamente implican una correlación con el fenotipo (Khan et al., 2019). Es así que en la actualidad se están utilizando nuevas metodologías como el studio de los polimorfismos puntuales, conocido como SNPs por su sigla en inglés (Single Nucleotide Polymorphism)(Calarco et al., 2018) lo que permite no solo caracterizar genéticamente las cepas sino relacionarlas con sus diferentes fenotipos (Calarco et al., 2018; Calarco and Ellis, 2019, 2020) .

1.6 Transmisión y sintomatología de la enfermedad

Las principales formas de transmisión de *N. caninum* al ganado bovino son la forma horizontal y la vertical. La transmisión horizontal, también denominada postnatal, tiene lugar mediante la ingestión de agua o alimento contaminado con ooquistes eliminados por el hospedador definitivo generalmente el perro (Dubey et al., 2007a), ya que la transmisión vertical o transplacentaria se produce cuando en una hembra gestante infectada, los taquizoítos se replican a nivel de placenta y logran invadir e infectar al feto (Figura 3)(Dubey et al., 1992). La transmisión transplacentaria o congénita es la vía de transmisión más eficiente del parásito, ya que se han descrito porcentajes de transmisión que oscilan entre el 40 y el 95% (Dubey et al., 2007a), jugando un papel fundamental en la propagación y mantenimiento de la enfermedad en un rebaño (Dubey et al., 2007a). Además, una hembra congénitamente infectada puede transmitir la infección a su progenie, repetidamente, en diferentes gestaciones (Boulton et al., 1995; Guy et al., 2001; Moloney et al., 2017). La transmisión transplacentaria de *N. caninum* al feto puede darse de dos maneras: por transmisión exógena o transmisión endógena (Trees and Williams, 2005). Estos modos de transmisión presentan consecuencias patogénicas y epidemiológicas diferentes (Hall et al., 2005).

La transmisión transplacentaria endógena ocurre tras la recrudescencia de una infección crónica durante la gestación en una hembra persistentemente infectada (Trees and Williams, 2005). En cambio, la transmisión transplacentaria exógena se presenta en vacas que adquieren la infección por primera vez por el consumo de ooquistes esporulados durante la gestación, es decir, transmitiendo la infección a su descendencia como consecuencia de la infección aguda (Dubey et al., 2007a). Las granjas infectadas crónicamente presentan abortos de manera endémica, como consecuencia de una transmisión transplacentaria endógena (Dubey and Schares, 2011). Por el contrario, las granjas que sufren una primera exposición a la infección por el contacto con ooquistes esporulados durante la gestación, presentan un brote de abortos (30-57%) debidos a una transmisión transplacentaria exógena (Dubey et al., 2007a; Piergili Fioretti et al., 2003; Trees and Williams, 2005). En esta línea, la transmisión transplacentaria endógena aparece como el modo de transmisión predominante en muchos establecimientos en los que las vacas se encuentran crónicamente infectadas. Existe controversia en cuanto a la relevancia de la transmisión transplacentaria exógena para dar lugar a infecciones crónicas (McCann et al., 2007; Williams et al., 2009). Se observó que las tasas de transmisión vertical disminuyen con la edad y si se le suma que la transmisión transplacentaria no ocurre en el 100% de los casos, se traduce en que, sin una fuente externa de infección (transmisión horizontal), la enfermedad tendería a desaparecer con el paso de las generaciones sucesivas (Dubey et al., 2007a). Por tanto, la transmisión transplacentaria exógena y endógena parecen complementarse para evitar este fenómeno generando una situación epidemiológica compleja (Dubey et al., 2007a).

La infección que genera *N. caninum* en el ganado bovino no gestante es, generalmente, asintomática, mientras que en animales gestantes tiene como signo clínico más relevante el aborto (Dubey et al., 2006). Si bien el aborto puede ocurrir a partir del tercer mes de gestación, suele

observarse con más frecuencia entre los 5 y 7 meses (Dubey et al., 2007a). Si la infección ocurre en el primer tercio de la gestación, el feto suele ser reabsorbido y se observa una repetición del celo (Almería and López-Gatius, 2013). No obstante, no se ha encontrado una asociación entre la infección por *N. caninum* y el fallo reproductivo temprano (Almería et al., 2010).

Por otra parte, si la muerte fetal se produce entre los meses 3 y 8 de gestación, el feto suele ser eliminado presentando una autolisis moderada. Sin embargo, algunos fetos que mueren antes del quinto mes podrían momificarse y quedar retenidos en el útero durante meses (Almería and López-Gatius, 2013; Dubey, 2005). Si la infección ocurre en etapas de la gestación más avanzadas, por ejemplo a partir del quinto mes, disminuye el riesgo de muerte fetal y el signo más frecuente será el nacimiento de terneros sanos pero congénitamente infectados (Trees and Williams, 2005). En algunos casos pueden observarse terneros infectados muy débiles con signos clínicos neurológicos, que van desde una ligera incoordinación hasta una parálisis completa. En los casos más graves, se puede observar malformaciones en la espina dorsal, hidrocefalia o neumonía (Dubey et al., 1992). Estos signos suelen aparecer en las primeras semanas de vida, causando parálisis total y ocasionando la muerte del animal (Dubey, 2005).

A nivel de la placenta se suelen observar focos de necrosis y zonas de intensa inflamación con infiltración de células mononucleares que, en procesos avanzados, pueden progresar hacia la regeneración conjuntiva con hiperplasia, fibrosis e incluso calcificación de los focos necróticos (Maley et al., 2003).

Las lesiones en el feto y en el ternero congénitamente infectado se encuentran mayoritariamente en el SNC. Éstas consisten en áreas multifocales de infiltración linfocitaria, con frecuencia alrededor de vasos sanguíneos (manguitos perivasculares), microgliosis y presencia de astrocitos, pudiendo existir zonas centrales de necrosis e, incluso, mineralización (Dubey and

Lindsay, 1996). Este tipo de inflamación no supurativa también se observa en el corazón e hígado y con menor frecuencia en el riñón, músculo esquelético o pulmón (Dubey et al., 2006). En cambio, en los terneros congénitamente infectados sin signos clínicos y en animales adultos también es posible encontrar lesiones, aunque con menor frecuencia y magnitud, restringiéndose principalmente al SNC (Barr et al., 1994).

1.7 Patogenia e inmunidad

Durante la parasitemia, que puede tener su origen en una primoinfección o, más comúnmente, en una reactivación de una infección crónica durante la gestación, los taquizoítos se diseminan por el organismo e invaden diferentes tejidos, siendo capaces de atravesar la barrera placentaria y llegar al feto (Dubey et al., 2006). Si bien los mecanismos producidos por el parásito para generar la muerte del feto no están del todo claros, uno de estos mecanismos que originaría graves lesiones afectando la supervivencia fetal puede ser la acción directa del parásito debido a la invasión y multiplicación en los tejidos placentarios y fetales. El otro mecanismo asociado al aborto pueden ser las lesiones en la placenta que podrían provocar un insuficiente intercambio de oxígeno y nutrientes al feto, ya sea directamente por la multiplicación de los taquizoítos en la misma o por la respuesta inmunitaria materna de tipo inflamatorio desencadenada como respuesta a la infección (Dubey et al., 2006). Por otro lado, una vez que el parásito invade la placenta y el feto, las diferentes consecuencias de la infección van a depender de diversos factores relacionados tanto con el parásito como con el propio hospedador, siendo los más importantes el periodo de gestación en el cual se produce la infección, lo que está directamente relacionado con la inmunidad materna y fetal (Dubey et al., 2006). La supervivencia del feto depende en gran medida del estado

de madurez de su sistema inmunitario y de su capacidad para hacer frente a la infección. Por tanto, cuanto más temprano se produzca la infección durante la gestación, más graves serán las consecuencias (Innes et al., 2000). Durante el primer trimestre de gestación el feto es altamente sensible a la infección, puesto que los órganos linfoides (timo, bazo y ganglios linfáticos periféricos) se encuentran aún en formación (Trees and Williams, 2005). Estos órganos comienzan a reconocer y responder frente a los agentes patógenos durante el segundo tercio de la gestación(Buxton et al., 2002). De esta forma, en el primer trimestre de gestación el feto es excepcionalmente vulnerable a la infección por *N. caninum* y con poca probabilidad sobrevivirá, mientras que en el segundo tercio de gestación el feto puede desarrollar una respuesta inmunitaria frente a la infección que puede o no ser suficiente para su control (Dubey et al., 2006; Maley et al., 2003). En el tercer trimestre, el feto ya se considera inmunocompetente y podría controlar la multiplicación del parásito y limitar el desarrollo de lesiones, permitiendo su supervivencia(Dubey and Schares, 2011), por lo cual, la infección en el último tercio de gestación comúnmente da lugar al nacimiento de terneros asintomáticos congénitamente infectados (Guy et al., 2001; McCann et al., 2007).

La infección por *N. caninum* en el ganado bovino desencadena una combinación de mecanismos de inmunidad innata y adquirida que intentan controlar conjuntamente la diseminación del parásito en el organismo (Bartley et al., 2013; Maley et al., 2003). Dichos mecanismos se ponen en funcionamiento en los primeros momentos tras la infección, activándose componentes de la inmunidad innata como células dendríticas, células NK (Natural Killer) y macrófagos. Estos tipos celulares actúan como primera línea de defensa, destruyendo las células infectadas por el parásito y liberando citoquinas del tipo IL-12 e IFN-γ (Almería et al., 2017; Dion et al., 2011). El reconocimiento del parásito por parte de las células presentadoras de antígeno y la

producción temprana de estas citoquinas es fundamental para el control de la proliferación de los taquizoítos en las primeras fases de la infección. Por otro lado, el papel de los receptores tipo Toll (Toll-like receptors, TLR) parece ser relevante durante las fases iniciales de la infección, ya que estos receptores se encuentran presentes en las células presentadoras de antígeno, así como en las células dendríticas y los linfocitos B (Bartley et al., 2013). Cabe destacar la importancia de esta primera respuesta innata e inespecífica como paso previo a la activación de los linfocitos y, por tanto, al consiguiente desarrollo de una respuesta inmunitaria específica con memoria inmunológica frente a *N. caninum*.

Al tratarse de un parásito intracelular la respuesta mediada por células es el componente más importante para la protección (Hemphill, 1999). El control de la infección para este parásito se evidencia una respuesta inmunitaria adquirida tipo Th1, donde están implicadas diferentes citoquinas proinflamatorias, principalmente IFN-γ, y poblaciones de células T (Dion et al., 2011; Dubey et al., 2006). La importancia del IFN-γ se ha puesto de manifiesto en varios estudios *in vitr*o e *in vivo*, donde se ha demostrado la inhibición de la multiplicación de *N. caninum* en cultivo celular por adición de IFN-γ al medio o en modelos animales (Correia et al., 2015; Nishikawa et al., 2001; Teixeira et al., 2016). A nivel celular, las células T CD4+ son activadas en presencia del IFN-γ en las primeras fases de la infección, orientando la respuesta inmunitaria hacia una de tipo Th1, activándose a su vez los linfocitos T CD8+. Estas células poseen actividad citotóxica y desempeñan un papel importante durante la fase aguda de la infección, ya que actúan limitando la diseminación del parásito y secretando IFN-γ (Correia et al., 2015). Sin embargo, en fases más avanzadas, los linfocitos T CD4+ parecen tener un papel más importante en la protección frente a *N. caninum*, pudiendo mediar en la lisis directa de células infectadas (Bartley et al., 2012). Por otro lado, los macrófagos infectados también pueden activar los linfocitos T CD4+ que expresarán

IL-17, una citoquina proinflamatoria reguladora que actúa conjuntamente con el IFN-γ (Flynn and Marshall, 2011). En conjunto, la respuesta Th1 es capaz de destruir las células infectadas por el parásito, además de inhibir su proliferación por medio de la secreción de IFN-γ y TNF-α y bloquear la invasión de nuevas células a través de los anticuerpos generados frente a los taquizoítos (Innes et al., 2000).

Por último, el papel de la respuesta inmunitaria humoral en el control de la neosporosis es todavía confuso. Es posible que los anticuerpos generados específicamente frente al parásito tengan varias funciones, como la opsonización de parásitos extracelulares con la consiguiente fagocitosis por parte de los macrófagos, tal y como ocurre en la toxoplasmosis (Sibley et al., 1985). Se registró que los niveles de anticuerpos frente al parásito pueden variar a lo largo de la gestación (Almería et al., 2009; Nogareda et al., 2007), lo que puede tener relación con la propia actividad proliferativa del parásito (Bartley et al., 2012). Sin embargo, los anticuerpos producidos contra el parásito son una buena herramienta para el diagnóstico de la enfermedad (McAllister, 2016).

1.8 Diagnóstico

En la actualidad existen varias técnicas de diagnóstico para detectar la infección por *N. caninum* (McAllister, 2016). Sin embargo, ya que los animales mantienen la infección de por vida, las técnicas más útiles para el diagnóstico de la enfermedad están basadas en la detección de anticuerpos específicos frente al parásito en suero. En este sentido se han desarrollado diferentes

técnicas de diagnóstico serológico, como la inmunofluorescencia indirecta (IFI) y el ensayo de inmunoabsorción ligado a enzima (ELISA) (McAllister, 2016). Estas pruebas son aplicables en animales adultos y recién nacidos, siempre y cuando se realice sobre sueros pre-calostrales. Además, existen ELISAs de avidez que son capaces de diferenciar infecciones recientes de crónicas (Björkman et al., 1997; Dubey et al., 2007a). Asimismo, se ha logrado la adaptación del ELISA en muestras de leche, mostrando resultados equiparables a los de suero, con las ventajas añadidas de tener menores costos y manejo del animal (Schares et al., 2004). También se ha demostrado que cuando se evalúan los diferentes métodos serológicos de diagnóstico, la sensibilidad y especificidad de las diferentes técnicas no varían en la detección del parásito (Campero et al., 2015).

Si bien las técnicas serológicas son el "gold standard" para la detección de *N. caninum*, las técnicas moleculares han tomado un papel crucial para la identificación del parásito tanto en fetos abortados como en tejidos de hospederos intermediarios (McAllister, 2016). En este sentido en presencia de un brote de abortos no solo se debe realizar el seguimiento serológico, sino también se debe acompañar de la aplicación de técnicas moleculares para la de detección del parásito en los tejidos de fetos abortados (cerebro principalmente) y la determinación de la existencia de lesiones compatibles en dichos tejidos (Dubey et al., 2007a; McAllister, 2016). Las técnicas moleculares como por ejemplo la reacción en cadena de la polimerasa (PCR) utilizadas tienen muy buena especificidad y sensibilidad y generalmente amplifican fragmentos genómicos conservados (Al-Qassab et al., 2010b; Yamage et al., 1996).

1.9 Profilaxis

En la actualidad, la única opción existente para el control de la infección en el ganado bovino se basa principalmente en la implementación de medidas de manejo a nivel del rodeo, ya que la quimioterapia e inmunoprofilaxis se encuentran aún bajo estudio, aunque se presentan como alternativas potenciales para el futuro (Sánchez-Sánchez et al., 2018).

La implementación de protocolos de bioseguridad en los establecimientos, medidas higiénicas y prácticas de manejo deben ser adoptadas para reducir el nivel de contaminación ambiental con ooquistes de *N. caninum*, así como la introducción de animales crónicamente infectados en el rodeo (Reichel et al., 2014). Entre las prácticas de bioseguridad que se deben realizar para lograr este objetivo, se destacan: limitar el acceso de perros a las áreas donde se encuentran los rumiantes y las áreas de almacenamiento de alimentos y suministro de agua; quitar rápidamente las placentas o los materiales fetales en la eventualidad de un aborto; desechar adecuadamente el ganado muerto; y establecer el control de roedores (Sánchez-Sánchez et al., 2018). En cuanto al manejo de las prácticas reproductivas antes de considerar la inseminación artificial o el uso de transferencia de embriones se recomienda el diagnóstico serológico de las madres para establecer la presencia de una infección crónica por *N. caninum* (Reichel et al., 2014). A pesar de estar adecuadamente diseñadas y practicadas meticulosamente, a nivel mundial, estas medidas de control por sí solas no son rentables ni completamente efectivas para eliminar la neosporosis de un rodeo, y es necesario complementarlas con un enfoque inmuno-quimioterapéutico (Hemphill et al., 2016; McAllister, 2016).

Con respecto a los fármacos frente a la neosporosis bovina, hasta la fecha no existen compuestos efectivos y seguros disponibles en el mercado. Además, aunque alguno de ellos ha mostrado recientemente resultados prometedores en ensayos *in vitro* y en modelos animales de

laboratorio (Müller and Hemphill, 2011), muchos necesitan de largos periodos de administración, lo que probablemente produciría residuos en carne y leche o periodos de espera poco rentables para la ganadería (Dubey et al., 2007a).

Debido a que el manejo y control del mismo es económicamente muy costoso y llevaría muchos años disminuir significativamente la incidencia del parásito en los establecimientos, la necesidad de desarrollar una vacuna constituye un consenso a nivel internacional (Horcajo et al., 2016), y se considera actualmente como la opción económicamente más viable para el control de la enfermedad en explotaciones con una elevada prevalencia (Weber et al., 2013). Durante varios años una vacuna comercial frente a la neosporosis bovina basada en un lisado de taquizoítos (Bovilis NeoguardTM) estuvo disponible en algunos países (Barling et al., 2003). Dicha vacuna mostró una protección moderada en pruebas de campo, con una reducción del aborto cercana al 50% (Romero et al., 2004). Sin embargo, ensayos recientes han revelado grandes diferencias en la eficacia a nivel de granja, mostrando cierto grado de protección frente a la transmisión horizontal, pero no vertical, llegándose incluso a sugerir que la propia vacunación podría incrementar el riesgo de muerte fetal temprana en las vacas inmunizadas (Weston et al., 2012).

En este sentido son varias las estrategias para generar nuevas vacunas para la protección del ganado contra este patógeno, desde las vacunas atenuadas con cepas avirulentas pasando por vacunas inactivadas (lisado de taquizoitos)(Aguado-Martínez et al., 2017; Marugan-Hernandez, 2017). Las nuevas metodologías también son usadas en este sentido como la generación de proteínas recombinantes para la realización de diferentes antígenos o la aplicación de métodos moleculares para la edición génica de los parásitos y producir cepas atenuadas (Bengoa-Luoni et al., 2019; Jia et al., 2013; Xu et al., 2019).

2.0 Hipóteis y Objetivos

2.1 Hipótesis

El aislamiento de cepas circulantes de *Neospora caninum*, y su estudio comparativo a nivel genotípico y fenotípico, tanto con cepas de referencia como con aislados de todos los continentes , permitirá profundizar en el conocimiento de la biología de este parásito, así como avanzar en el desarrollo de nuevas estrategias para el control y erradicación de dicha enfermedad.

2.2 Objetivo General

Caracterización genética y fenotípica de las cepas circulantes de *Neospora caninum* en Uruguay, y su análisis comparativo con cepas de referencia y aislamientos a nivel mundial.

2.3 Objetivos específicos

1) Aislamiento de cepas circulantes de *N. caninum*.
2) Caracterización genética de las cepas aisladas de *N. caninum* mediante marcadores moleculares.
3) Caracterización fenotípica las cepas aisladas de *N. caninum*, tanto *"in vitro"* como *"in vivo"*.

3.0 Aislamiento y caracterización molecular de cuatro nuevas cepas de *Neospora caninum*

En virtud de la importancia de la producción bovina en la economía del país nos propusimos aislar y caracterizar genéticamente las diferentes cepas de *Neospora caninum* que circulan en nuestro territorio, para generar herramientas locales para su diagnóstico y así aplicar medidas de prevención contra esta enfermedad.

Sin embargo, como mencionamos anteriormente, se registran muy pocos aislados a nivel regional y mundial de *N. caninum*. Esto se debe, en parte, a que el aislamiento de cepas implica un trabajo de coordinación y colaboración interdisciplinario, que involucra el contacto estrecho entre investigadores básicos con productores y veterinarios. Al comienzo de esta tesis doctoral los únicos países en la región habían logrado aislar exitosamente el parásito eran Brasil y Argentina (3 aislados y 1 aislado, respectivamente). Si bien en Uruguay se había detectado la presencia del parásito mediante métodos serológicos, no se contaba con aislamientos autóctonos ni con datos sobre la epidemiología molecular del mismo.

Las preguntas que nos hicimos fueron: ¿Somos capaces de realizar nuestros propios aislamientos de N. caninum teniendo en cuenta la complejidad logística que implica? ¿Las cepas que circulan en Uruguay son las mismas que circulan en la región? En virtud de estas preguntamos empezamos a generar el conocimiento para responder las mismas.

Para llevar adelante este trabajo fue imprescindible la conformación de un grupo interdisciplinario donde participaron distintas instituciones. Por un lado el Institut Pasteur de Montevideo, donde se desarrolló esta tesis doctoral, en el Laboratorio de Interacciones Hospedero

Patógeno, que aportó la infraestructura y la experticia para el desarrollo de los estudios celulares y moleculares. También fue fundamental la participación de la Unidad de Animales Transgénicos y de Experimentación (UATE), donde se pudo establecer el modelo murino de infección para el aislamiento y mantenimiento del parásito. Finalmente, la Plataforma de Salud Animal del Instituto Nacional de Investigaciones Agropecuaria (INIA) La Estanzuela, fue clave, tanto en el aporte de recursos humanos de alto nivel y de diferentes profesiones (veterinarios, biólogos, bioquímicos, técnico de laboratorios entre otros) como en la infraestructura del tambo experimental, en donde se detectaron las vacas preñadas seropositivas, así como los terneros congénitamente infectados para realizar los aislamientos.

Para llevar adelante los aislamientos tuvimos que desarrollar herramientas que hasta el momento no se encontraban funcionando en el país. Si bien el laboratorio donde se desarrolló esta tesis de doctorado, se trabajaba con diferentes parásitos, no existían antecedentes con Apicomplejos, por lo que ésta línea era totalmente nueva. En la primera etapa se adquirieron en el exterior cepas de referencia de *N. caninum* (NcLiverpool y NcBahía), y se pusieron a funcionar los protocolos de co-cultivos con células de mamífero, lo cual significó el primer hito de esta tesis doctoral. Cabe destacar que paralelamente se estableció el cultivo de *Toxoplasma gondii*, para los posteriores estudios de diagnóstico diferencial. Se logró optimizar por primera vez en el país diferentes métodos de extracción de muestras biológicas y métodos moleculares, con el fin de detectar y caracterizar el parásito. Paralelamente y con el apoyo de la unidad de Animales Transgénico y de Experimentación, se estableció el modelo animal de infección con *Neospora caninum*. Para ello ensayamos tres modelos: ratones Balb/c, Nude, y knock-out para el gen de interferón gamma, seleccionando finalmente estos últimos, que son los que más se han reportado en la literatura para llevar a cabo aislamientos, porque al ser inmunodeficientes y no resolver la

infección, generan altas parasitemias, pero a la vez generan una respuesta de anticuerpos que permite detectar el curso de la misma (a diferencia de los ratones Nude en los que no puede detectarse serología positiva). Este conjunto de herramientas desarrolladas en la primera etapa de esta tesis de doctorado, permitió crear todas las condiciones necesarias para intentar el aislamiento de cepas uruguayas. Así mismo, desarrollamos colaboraciones con laboratorios de referencia a nivel mundial, quienes contaban con amplia experiencia en aislamientos. Los laboratorios dirigidos por el Dr. Luis Miguel Ortega-Mora, el Dr. Dadin Prando Moore y la Dra. Cecilia Venturini, fueron instrumentales para la puesta a punto de protocolos.

Conjuntamente con la Plataforma Salud Animal y el personal del tambo de INIA La Estanzuela, se organizó un plan de trabajo donde se detectaron vacas preñadas positivas para *N. caninum* y se realizó el seguimiento durante el periodo de preñez. Una vez llegado el momento de parición se identificaron terneros machos congénitamente infectados candidatos para llevar adelante el aislamiento.

Como resultado de esta primera parte logramos obtener cuatro aislados de *Neospora caninum* autóctonos. En una siguiente etapa, logramos caracterizar genéticamente a estos aislados. Para ello utilizamos marcadores moleculares (microsatélites) que nos permitieron compararlos con las cepas circulantes en la región y el mundo.

Finalmente, logramos identificar por técnicas moleculares, genotipos parciales de cepas de *N. caninum* presentes en especímenes obtenidos a partir de abortos bovinos de varias regiones del país, y compáralos con los genotipos identificados en los aislamientos. Este análisis nos llevó a concluir que existe una gran variabilidad genética en las cepas uruguayas. Al realizar un estudio con cepas regionales y mundiales logramos agrupar las diferentes cepas de *N. caninum* en seis grande grupos.

Los resultados obtenidos fueron publicados en el artículo que se incluye a continuación, en la revista Parasitology Research en 2019. (**Cabrera A**, Berná L, Fresia P, Silveira C, Macias Rioseco M, Pritsch O, Riet-Correa F, Giannitti F, Francia ME*,Robello C*. (2019). Isolation and molecular characterization of four novel *Neospora caninum* strains. Parasitology Research (2019) 118(12):3535-3542)

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GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Isolation and molecular characterization of four novel *Neospora caninum* strains

Andres Cabrera[1] · Pablo Fresia[2] · Luisa Berná[1] · Caroline Silveira[3] · Melissa Macías-Rioseco[3] · Ana Paula Arevalo[4] · Martina Crispo[4] · Otto Pritsch[5,6] · Franklin Riet-Correa[3] · Federico Giannitti[3,7] · Maria E. Francia[1,8,9] · Carlos Robello[1,10]

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Abstract

Neospora caninum causes neosporosis, a leading cause of bovine abortion worldwide. Uruguay is a developing economy in South America that produces milk to feed seven times its population annually. Naturally, dairy production is paramount to the country's economy, and bovine reproductive failure impacts it profoundly. Recent studies demonstrated that the vast majority of infectious abortions in dairy cows are caused by *N. caninum*. To delve into the local situation and contextualize it within the international standing, we set out to characterize the Uruguayan *N. caninum* strains. For this, we isolated four distinct strains and determined by microsatellite typing that these represent three unique genetic lineages, distinct from those reported previously in the region or elsewhere. An unbiased analysis of the current worldwide genetic diversity of *N. caninum* strains known, whereby six *typing clusters* can be resolved, revealed that three of the four Uruguayan strains group closely with regional strains from Argentina and Brazil. The remaining strain groups in an unrelated genetic cluster, suggesting multiple origins of the local strains. Microsatellite typing of *N. caninum* DNA from fetuses opportunistically collected from local dairy farms correlated more often with one of the isolates. Overall, our results contribute to further understanding of genetic diversity among strains of *N. caninum* both regionally and worldwide.

Keywords Apicomplexa · Bovine abortion · *Neospora* · Microsatellite · Genetic diversity · Animal health

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✉ Maria E. Francia
mfrancia@pasteur.edu.uy

✉ Carlos Robello
robello@pasteur.edu.uy

[1] Laboratory of Host Pathogen Interactions-UBM, Institut Pasteur de Montevideo, Montevideo Uruguay

[2] Bioinformatics Unit, Institut Pasteur de Montevideo, Montevideo Uruguay

[3] Plataforma de Salud Animal, Estación Experimental INIA La Estanzuela, Instituto Nacional de Investigación Agropecuaria (INIA), Ruta 50 km 11 Colonia del Sacramento Uruguay

[4] Transgenic and Experimental Animal Unit, Institut Pasteur de Montevideo, Montevideo Uruguay

[5] Immunovirology Unit, Institut Pasteur de Montevideo, Montevideo Uruguay

[6] Departamento de Inmunobiología, Facultad de Medicina, Universidad de la República, Montevideo Uruguay

[7] Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, Saint Paul MN USA

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

[9] Departamento de Parasitologia y Micologia, Facultad de Medicina, Universidad de la República, Montevideo Uruguay

[10] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo Uruguay

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Abbreviations

AMOVA	Analysis of molecular variance
DAPC	Discriminant analysis of principal components
DMEM	Dulbecco's modified Eagle's medium
HFF	Human foreskin fibroblast
MS	Microsatellite
PCA	Principal component analysis

Introduction

Neospora caninum is an obligate intracellular protozoan parasite of the phylum Apicomplexa, which preferably infects cattle as intermediate hosts, causing neosporosis, one of the main causes of cattle abortion and reproductive failure worldwide (Dubey and Schares 2011). Cattle acquire the parasite by ingesting oocysts shed in feces by the definitive hosts, wild canids, or domestic dogs, developing, in most cases, a chronic asymptomatic disease (Dubey and Schares 2011). Canids become subsequently infected by ingestion of tissue cysts lodged in the intermediate hosts, hence perpetuating the transmission cycle (Cavalcante et al. 2011). *N. caninum* can also be transmitted transplacentally (Gondim et al. 2004). Infected fetuses can display irreversible tissue damage, resulting in fetal death and abortion, or be born asymptomatic and chronically infected. In chronically infected bovine herds, abortions are normally associated with transplacental transmission following reactivation of a dormant infection. Epidemic abortion outbreaks, known as "abortion storms," in naive pregnant cows can also occur and are normally associated with oocyst-derived infection (exogenous transplacental transmission) (Dubey and Schares 2011).

N. caninum abortion in livestock is estimated to generate millions of dollars in losses worldwide (Reichel et al. 2013). For instance, studies in the UK estimated that 12.5% of annual abortions in dairy cattle are caused by this agent (Davison et al. 1999). Meanwhile, in China, this number varies between 26 and 40% (Jia et al. 2014). In Argentina, *N. caninum* has been estimated to produce economic losses of up to 44 million USD (Moore et al. 2013). Uruguay produces milk to feed more than twenty million people annually, and the number of cows triples the human population. Thus, dairy production is of paramount importance to the country's economy. Seroepidemiological studies dated over 10 years ago estimated that virtually all dairy farms in Uruguay had at least one seropositive cow for *N. caninum* (Bañales et al. 2006; Piaggio 2006), and that about a third of the diagnosed abortions were caused by this agent (Easton 2006). Currently, the epidemiological situation has remained unchanged; a large share of current abortions in dairy cattle, diagnosed at the laboratory level, is caused by *N. caninum* (Macías-Rioseco M., personal communication[1]).

Previous studies have shown that genetic diversity exists among *N. caninum* isolates from geographically adjacent regions, including those present in South America (Al-Qassab et al. 2009; Regidor-Cerrillo et al. 2013). However, genetic diversity among *N. caninum* in South America is understudied with respect to worldwide diversity. More recently, an isolate from Argentina (Campero et al. 2015) and two novel Brazilian isolates (Locatelli Dittrich et al. 2018) have been reported. To deepen our understanding of both the national and regional situations, here we set out to characterize, at the molecular level, *N. caninum* isolates in Uruguay and to compare them with recent regional and worldwide isolates. We isolated four strains from congenitally infected dairy calves originating from three distinct geographical regions in Uruguay. Multilocus microsatellite typing of genomic DNA revealed a unique genetic pattern, resulting in the distribution of the Uruguayan isolates into two distinct genetic groups. Importantly, our results indicate that greater genetic variability exists among regional *N. caninum* populations than previously appreciated, contributing to further understanding this parasite's genetic diversity.

Material and methods

Strain isolation

To increase the chances of isolating distinct genetic backgrounds, we selected individuals from dairy farms with different neosporosis histories and from distinct geographical locations within the country's dairy-producing area. Three of the isolated strains (NcUru1–NcUru3) originated from *La Estanzuela*, in Colonia Department (Fig. S1A), an experimental dairy farm with approximately two hundred Holstein cows, located at the National Institute of Agricultural Research (INIA), whereby abortions occur sporadically. This herd presented chronically infected individuals. The seroprevalence of *N. caninum* in this herd is about 25%, and abortions have a sporadic occurrence. Here, we identified ten of thirty-six pregnant cows/heifers (28.5%) to be seropositive for *N. caninum* by ELISA. One of them had been recently acquired from a commercial dairy farm in the department of Soriano and was already pregnant when brought over to La Estanzuela (Fig. S1A). Six asymptomatic, congenitally infected male calves, with precolostral antibodies for *N. caninum*, were selected to pursue isolation, including the calf born to the heifer from Soriano. The isolate NcUru4 was derived from a dairy farm located in Durazno (Fig. S1A). This is a large farm with over ten thousand cows, which had experienced a laboratory-confirmed

[1] Macias-Rioseco, M. Instituto Nacional de Investigación Agropecuaria (INIA). Plataforma de Salud Animal. Contact: mmacias@inia.org.uy

epizootic outbreak of *N. caninum* abortion affecting several hundred dams. Here, four newborn asymptomatic male calves with precolostral antibodies to *N. caninum* were selected to pursue isolation. Pregnant cows were diagnosed positive for *N. caninum* using a commercial ELISA kit (PrioCHECK™ Bovine Neospora Ab 2.0 Serum Kit, Thermo Fisher Scientific, Waltham, MA, USA) and followed to term.

Precolostral serum samples were obtained from newborn calves, and *N. caninum* antibodies were determined by the same ELISA kit. Seropositive calves were selected to attempt parasite isolation. For a scheme, refer to Supplementary Figure 1. Parasite isolation was carried out following the procedure described previously (Pena et al. 2007; Rojo-Montejo et al. 2009; Campero et al. 2015). In short, brain samples of the congenitally infected calves were homogenized in a phosphate-buffered saline (PBS) solution containing antibiotics, DNA was extracted, and PCR assay was carried out to identify areas of the brain harboring *N. caninum* DNA. PCR-positive homogenates were processed and inoculated into interferon-γ knockout mice B6.129S7-Ifngtm1Ts/J (Jackson Laboratory, Bar Harbor, Maine, USA) by intraperitoneal injection (200 μl). Mice were evaluated daily for the onset of clinical signs compatible with neosporosis (ataxia, forelimb paralysis, emaciation, and/or lethargy). Symptomatic mice were humanly euthanized at the post-inoculation times indicated in Supplementary Material Figure 2. Brain samples were aseptically obtained, tested by PCR to identify *N. caninum*-infected regions, and inoculated onto human foreskin fibroblasts (HFF-1, SCRC-1041) (ATCC, Manassas, VA, USA). Parasites were observed in tissue culture, at the times indicated in Supplementary Material Figure 2, for each strain.

All experiments involving animals were done following pre-approved protocols by institutional ethic committees (CEUA no. 010-17) at the Institut Pasteur de Montevideo or the National Institute for Agricultural Research (INIA).

Cell culture and strain maintenance

Cell culture was maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) solution and 5% fetal bovine serum (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO_2. All isolates were serially passaged in human foreskin fibroblasts (HFFs). Isolates were cryopreserved in liquid nitrogen on 10% DMSO (Sigma-Aldrich, Merck, Darmstadt, Germany).

DNA extraction, polymerase chain reaction, and microsatellite typing

Genomic DNA was extracted using a commercial kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. *N. caninum*-specific detection by PCR was carried out by amplifying the *N. caninum*-specific gene Nc5, using primers Np6 and Np21, as described previously (Yamage et al. 1996). Coccidian and *Toxoplasma gondii*-specific DNA detection was carried out as described previously (Michael et al. 1996; Homan et al. 2000). A mammalian nuclear gene was amplified as a DNA extraction control, as described previously (Murphy et al. 2001). Microsatellite analysis was performed on *N. caninum* PCR–positive samples extracted from isolated strains in vitro and on bovine samples. The genetic markers MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21 were amplified using specific primers and PCR conditions previously described (Regidor-Cerrillo et al. 2013). PCR products were analyzed by 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, Carlsbad, CA, USA), purified (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany), and sequenced in house. Sequences were analyzed using BioEdit Sequence Alignment Editor v.7.0.1 software (Ibis Therapeutics, Carlsbad, CA, USA). Allele assignment was performed as described previously (Regidor-Cerrillo et al. 2013). Fifteen aborted fetuses recovered from the field, with *N. caninum*-compatible lesions or with an undiagnosed cause of abortion, were processed for DNA extraction and subjected to PCR analysis for *N. caninum* DNA detection, microsatellite amplification, and analysis, as described above. All fetuses came from the dairy-producing region of the country (Fig. 1a). Particularly, 4 of them (Fig. 1a, samples 8, 10, 12, and 14) originated from regions adjacent to the dairy farm where NcUru1 and NcUru2 were isolated. Sample 7 originated in the same region as NcUru3. The remaining 10 samples were obtained from other localities, as shown in Fig. 1 a.

Immunofluorescence assay

Immunofluorescence assay of intracellular tachyzoites grown on HFFs was carried out using a commercial anti-*N. caninum* antiserum raised in goat (1:100) (VMRD, Pullman, WA, USA). Donkey anti-goat IgG H&L-FITC conjugated (Abcam, Cambridge, UK) was used at a 1:1000 dilution as secondary antibody. Acquisition was carried out in an epifluorescence microscope (Olympus Life Science, Tokyo, Japan) using a × 100 oil objective.

Histopathology and immunohistochemistry

Brain samples from the *N. caninum*-congenitally infected neonate calves and liver samples from the inoculated mice were collected postmortem, immersion fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, sectioned at 4–5μm, mounted on glass slides, and stained with hematoxylin and eosin for routine histologic examination. Additionally, selected formalin-fixed paraffin-embedded (FFPE) sections of the liver from the mice were processed for *N. caninum* antigen detection by immunohistochemistry. Briefly, the slides were immersed in citrate buffer and heat-induced antigen retrieval was performed in

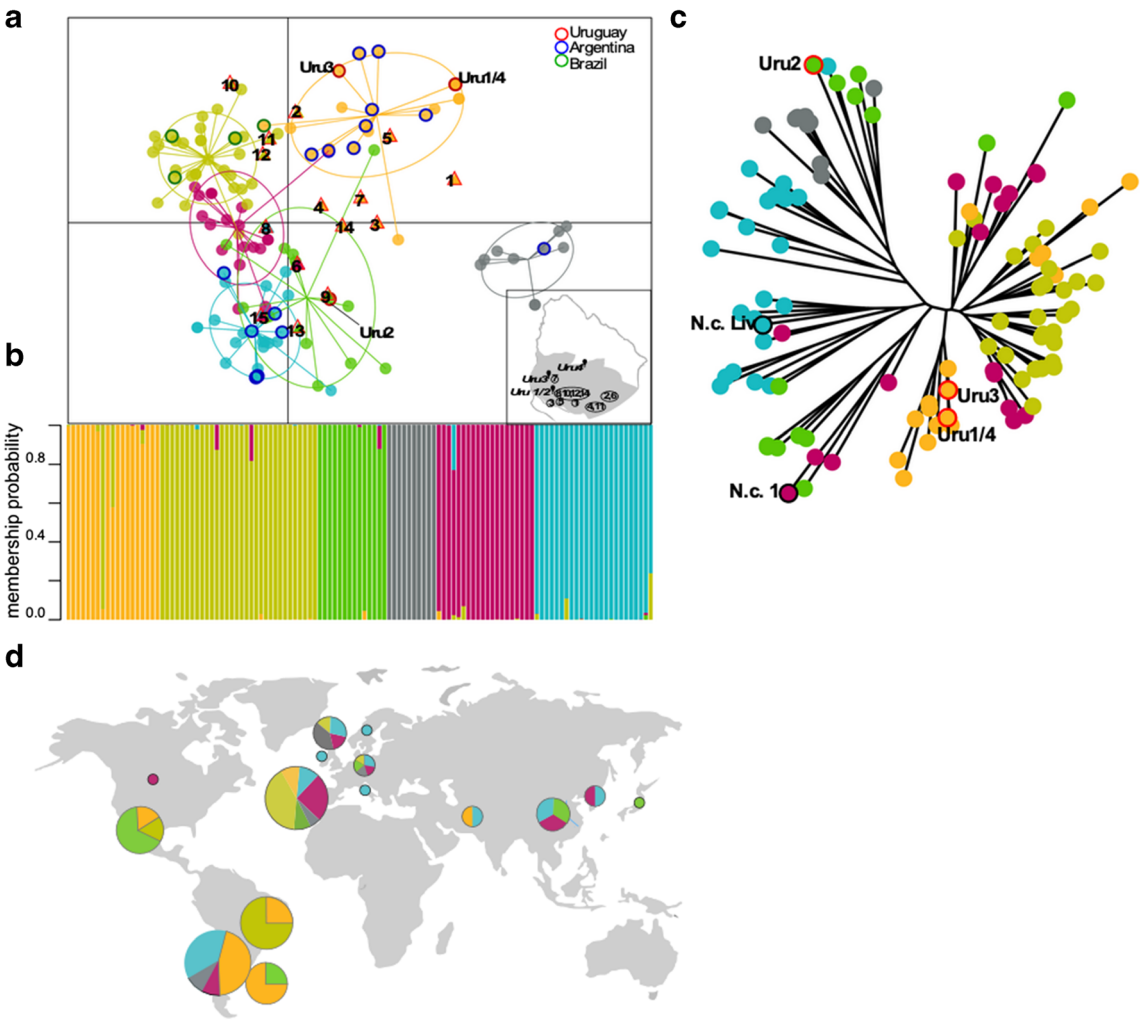

Fig. 1 **a** Strain assignment to a genetic group by discriminant analysis of principal components (DAPC). Strains were grouped according to their combination of 8 microsatellite alleles into 6 distinct genotypic groups. Argentinean strains are circled in blue, Brazilian strains in green, and Uruguayan strains in red. See Supplementary Table 1 for details on the strains used in this analysis. Strains identified in aborted fetuses were assigned to a given genetic group according to their combination of microsatellite alleles and are represented with a triangle. Each strain was color coded as per their assigned group and numbered to match its geographical origin specified in the inset. The probability of each strain belonging to a given group identified in abortion samples is detailed in Supplementary Table 2. The gray-shaded area represents the dairy-producing region of the country. **b** Likelihood of genotype assignment per strain. The likelihood of each strain to be assigned to a given genotype is shown. Color codes correspond to the ones used in **a**, for each group. **c** Neighbor-joining tree. Evolutionary distances among strains sampled are graphically represented. **d** Geographical distribution of genotypes. Genotypes are color coded to match those in **a**

a decloaking chamber (Biocare Medical, Pacheco, CA, USA) at 110 °C for 30 min, after quenching the endogenous peroxidase with 3% hydrogen peroxide for 15 min. Goat polyclonal antibody against *N. caninum* (VMRD, Pullman, WA, USA) was applied as a primary antibody for 60 min. Horse anti-goat IgG horseradish peroxidase (HRP)–labeled polymer (Vector Laboratories, Burlingame, CA, USA) was used as the detection system (30-min incubation), with 3-amino-9-ethylcarbazole (Dako, Santa Clara, CA, USA) as the chromogen substrate solution. Archived FFPE brain from a dog infected with *N. caninum* was used as a positive control. As negative controls, duplicate sections of all slides were processed as mentioned above, but the primary antibody was replaced by normal goat serum (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with hematoxylin and visualized under an Axio Scope.A1 trinocular optical microscope (Zeiss, Oberkochen, Germany), coupled with an Axiocam 512 digital camera (Zeiss, Oberkochen, Germany).

Principal component analysis and discriminant analysis of principal components

Genetic structure of samples from Argentina, Brazil, and Uruguay was first assessed by a principal component analysis (PCA). To perform the principal component analysis, a combination of nine microsatellites (MSs) was used to generate a

genetic distance matrix using the allele-sharing coefficient using *princomp* in R Studio (RStudio Team 2015). The nine MSs analyzed resulted from splitting MS10 in three independent satellites, as this previously showed better resolution. Argentinian and Brazilian strains were included (n = 25). The results were not affected by missing data, since the results did not change using NA or imputation by mean. Then, the discriminant analysis of principal components (DAPC) in the *adegenet* package (Jombart 2008), implemented in R, was used to investigate the genetic structure of samples from Asia, Europe, and the Americas. DAPC does not make assumptions of the Hardy–Weinberg equilibrium, so all microsatellite loci were included in the analysis. The optimal number of clusters was determined using the *find.clusters* function of adegenet by k-means clustering and run sequentially with an increasing value of k (k = 1 to k = 15), and the best number of clusters was chosen by the lowest value of the Bayesian information criterion (BIC) (Jombart et al. 2010). We ran the find.clusters with 1000 starting points and 1,000,000 iterations, and the results were consistently convergent over 10 independent trials. The assignment probability of the *N. caninum* strains isolated from abortions was estimated with the *predict.dapc* function over the previously identified clusters in the adegenet package (Jombart 2008). The distribution of the genetic variability within and between clusters, and Φ_{ST}, was estimated by the analysis of molecular variance (AMOVA) (Excoffier et al. 1992) in the *poppr* package (Kamvar et al. 2013) implemented in R. The genomic accumulation curve and neighbor-joining tree with Provesti's distance were done with the poppr package (Kamvar et al. 2013)

Results and discussion

Genetic characterization of four novel Uruguayan *N. caninum* isolates from congenitally infected calves

In order to characterize *N. caninum* isolates in Uruguay, we obtained four *in vitro* isolates herein referred to as four distinct isolates, NcUru1 and NcUru2 (from the region of Colonia), NcUru3 (from the region of Soriano), and NcUru4 (from the region of Durazno) (Fig. S1A). In short, *N. caninum* PCR–positive regions of the brain from congenitally infected calves were inoculated in IFN-γ-KO or nude mice. Tachyzoites were isolated post inoculation of PCR-positive mouse brain regions onto HFFs (Fig. S1B, 4 and Fig. S2). Species identity was confirmed by PCR (Fig. S1C).

MSs are highly variable molecular markers which allow genotyping individuals within a population, and the study of phylogenetic relationships (Field and Wills 1996). We characterized well-known MS markers on our in vitro isolates as to be able to compare their genetic diversity with previously characterized strains (Al-Qassab et al. 2009; Pedraza-Díaz et al. 2009; Rojo-Montejo et al. 2009; Regidor-Cerrillo et al. 2013; Campero et al. 2015, 2018; Medina-Esparza et al. 2016).

All, but NcUru1 and NcUru4, exhibited a distinct pattern of microsatellites, suggesting that they represented at least three distinct strains (Table 1). The only microsatellite allele shared among all four isolates was MS8, consisting of 13 repeats. This allele is also found in Brazilian isolates and has been detected in samples obtained from abortions caused by *N. caninum* in Argentina (Table 1). NcUru2 only shared MS8 with NcUru1, NcUru3, and NcUru4 and was distinct for all other MSs. Moreover, NcUru2 shared little identity at the MS level with previously reported isolates from the region, exhibiting a combination of MS7 and MS10 (13 and 6.14.7 repeats, respectively) which has not been described in regional strains (i.e., Argentina and Brazil) before. Moreover, its individual combination of microsatellites was unique and has not been previously reported. We speculate that this scenario could have emerged by the introduction to Uruguay of strains with two different genetic origins.

The *N. caninum* species groups in six typing clusters

We sought to determine how NcUru1–NcUru4 fit among the genetic variabilities reported for *N. caninum* strains identified worldwide (Table S1). By iterating all MS sequence combinations available (found in Table S1), a genotype accumulation curve revealed that a combination of 8 MSs was sufficient to achieve the maximum number of genotypes able to be resolved among unique individuals given a random sample of *n loci* (Fig. S4). Adding the genetic information available for 115 other worldwide strains, we ran DAPC that statistically supported the assemblage of six distinct genetic groups, arbitrarily named 1 through 6 (Fig. 1a and Fig. S5). Through AMOVA, we observed that ~ 30% of the variation was due to differences between clusters with Φ_{ST} = 0.3 ($p < 0.001$), and that ~ 70% was due to genetic variability within clusters. Cluster 2 grouped ~ 27% of the total samples, and cluster 6 (grouping ~ 20% of the total samples) was the most widely distributed, composed of strains originating in 10 different countries (Fig. 1a). As previously reported, this analysis showed a tendency for genotypes to group by geographical origin (Al-Qassab et al. 2009; Regidor-Cerrillo et al. 2013). At the continental scale, it was observed that ~ 63% of cluster 1 was from the Americas, and ~ 84% of cluster 2, 90% of cluster 4, 80% of cluster 5, and ~ 63% of cluster 6 were from Europe. Cluster 3 was distributed ~ 50% in the Americas and ~ 50% in Europe (Fig. 1c). Nonetheless, all six typing units were present in the Americas and Europe. Of note, cluster 2 was the only one not found in Asia. At a country level in South America, three of the four reported Brazilian isolates grouped together in cluster 2. Argentinean strains grouped in cluster 1 or 6, with a single exception in cluster 4. Neither Brazilian nor Argentinean strains were found elsewhere. Likewise, this

Table 1 Comparison of four Uruguayan *N. caninum* isolates with regional strains by microsatellite typing

STRAIN	ORIGIN	HOST	MICROSATELLITE MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21	SOURCE
N. c. Uru1	URUGUAY	Cattle	11	14	15	12	9.1	13	6.15.9	16	6	This study
N. c. Uru2			14	9	12	11	13		6.14.7	15	5	
N. c. Uru3			13	14	15	12	9.1		6.15.9	16	6	
N. c. Uru4			11									
N. c. 1	USA	Dog	12	12	12	17	16	16	7.12.9			Regidor-Cerrillo, 2013
N. c. Liv	UK		15	9	15	12	14	17	6.26.10			Regidor-Cerrillo, 2006
N. c. Goias	BRAZIL	Cattle	13	18	14	12	9.1	12	6.16.9	16	6	García-Melo, 2009 Regidor-Cerrillo, 2006 Oliveira, 2017 Locatelli, 2018
N. c. Bahia				13	15			14	5.14.9			
N. c. PSP1				19					6.14.10			
N. c. BNC-PR4			14	15				13	6.13.9			
N.c. ARG-04-1	ARGENTINA		13	17					6.15.8			Regidor-Cerrillo, 2013
N.c. ARG-04-2			11	10	11	11	NA	NA				
N.c. ARG-05-3			13		NA	13	10	14	6.12.7		5	
N.c. ARG-05-4				16	16	12	9.1	13	6.15.9		6	
N.c. ARG-05-5				9	13	11	15	16	6.17.10	16		
N.c. ARG-05-6									6.17.9			
N.c. ARG-05-7							14	13	6.18.10			
N.c. ARG-05-8				NA	16	12	9.1		5.15.9			
N.c. ARG-07-9				9	14	11	14	16	6.17.11			
N.c. ARG-07-10			11	14	15	12	9.1	12	6.15.9			
N.c. ARG-07-11			13	11	NA	NA	NA	16	6.16.9			
N.c. ARG-07-12				9	13	11	16		6.18.10			
N.c. ARG-07-13			11	NA	14	12	NA	12	6.15.9		NA	
N.c. ARG-08-14			13	9	16		16	13			6	
N.c. ARG-08-15			12	15	14		9.1	12				
N.c. ARG-08-16			13	14	NA		NA					
N.c. ARG-LP1			NA	16	16	13	10	NA				Campero, 2015
N.c. ARG-Goat		Goat	14	15		12	9.1	13	5.14.8		7	Campero, 2018

Microsatellite allele configuration of each Uruguayan strain is shown. The number of repeats within the microsatellite is shown. The microsatellite configurations previously characterized in regional isolates as well as in circulating *N. caninum* strains from aborted fetuses are shown. Commonly used *reference* strains N. c. Liverpool and Nc-1 are included. Coinciding alleles for each MS are shaded in the same color for clarity. Uncolored alleles appear only once for each column. *NA* denotes uncharacterized microsatellite sequences

analysis showed that the Uruguayan strains were found in two clusters: NcUru1, NcUru3, and NcUru4 in cluster 1 and NcUru2 in cluster 3. Interestingly, the latter cluster included strains identified in abortions, but no representative isolate has been reported. Lastly, much like the Argentinean "outlier" strain grouping in cluster 4, NcUru2 did not group with any other South American strain. Instead, it grouped with strains from Mexico, Spain, Germany, Japan, and China and was more closely related to the Argentinean strains present in cluster 6 than to its Uruguayan counterparts (Fig. 1a, Table S1). A neighbor-joining tree, based on Provesti's distance, supported the previous clustering analysis in which individuals that were grouped by DAPC tended to be more closely related (Fig. 1c). Incongruences observed between the tree and DAPC analysis were consistent with the degree of admixture among clusters, as shown by the membership probability plot (Fig. 1b).

The fact that three out of the four isolates clustered by geographical origin, while still grouping closely to genotypes present in Europe, may suggest that minimal genetic drift has occurred from founding strains originating from the founding cow import waves. This could indicate that strain mixes by sexual replication and recombination occurs at low frequency, or alternatively, that the strains experiencing recombination are rarely transmitted to cows.

Molecular examination of *N. caninum* strains causing bovine abortions reveals additional local variability

Lastly, we wondered whether our in vitro isolates were representative of strains causing abortions. To test this, we amplified and sequenced the same combination of

Table 2 Microsatellite typing of *N. caninum* strains found in aborted bovine fetuses

CASE	MICROSATELLITE MS4	MS5	MS6A	MS6B	MS7	MS8	MS10
1	11		12	*	*	13	6.15.9
2	13		*	12	9.1	14	
3			12		*	*	
4	*	*	*	*		13	*
5					9.1		6.15.9
6	12		12				
7	11		*	12		*	*
8	13	14	12		*		
9	*	*	*	11			
10	13	15	16	12	9.1	13	6.14.9
11		14	15				
12		15	16			*	
13	19	9	14	11	10	14	*
14	*	*	13	12	9.1	*	
15	12		*	*	10		

NcUru1/4 allele NcUru2 allele NcUru3 allele

Asterisk denotes an allele failed to be amplified by PCR

nine microsatellites, used to type the isolates, from 15 aborted fetuses, confirmed by PCR for the presence of *N. caninum*. Due to the variable states of conservation of the DNA in these samples, we succeeded in amplifying a variable number of MS per fetus (Pedraza-Díaz et al. 2009). MS12 and MS21 could not be amplified in any of the samples. Interestingly, the multiple combinations of MS identified suggest that the abortions were caused by multiple strains of *N. caninum*, and that we have not yet saturated our sampling, as clearly there is still a rich pool of uncharacterized genetic diversity among strains present in the country. Only samples 4, 5, 9, and 11 exhibited a combination, albeit incomplete, potentially compatible with our isolates, whereby only sample 9 could possibly correspond to an NcUru2 genotype (Table 2). On the other hand, 11 other strains detected seemed to differ from NcUru1 through NcUru4, from each other, and from previously reported regional isolates (Table 2 and Table S2).

Despite the validity of microsatellites as an epidemiological approximation, it is well established that MS variation does not correlate, *per se*, with phenotypic traits. This discordance is consistent with NcUru1 and NcUru4 being genetically identical by MS typing but isolated from farms with very different histories. NcUru1 originated from a dairy farm with a history of sporadic abortions. In stark contrast, NcUru4 was isolated from a dairy farm where approximately 700 abortions were registered within a weeks' time. Complete genomic sequences of multiple isolates from distinct geographical origins would be required to perform comprehensive functional inferences by mapping genes responsible for distinct virulence phenotypes.

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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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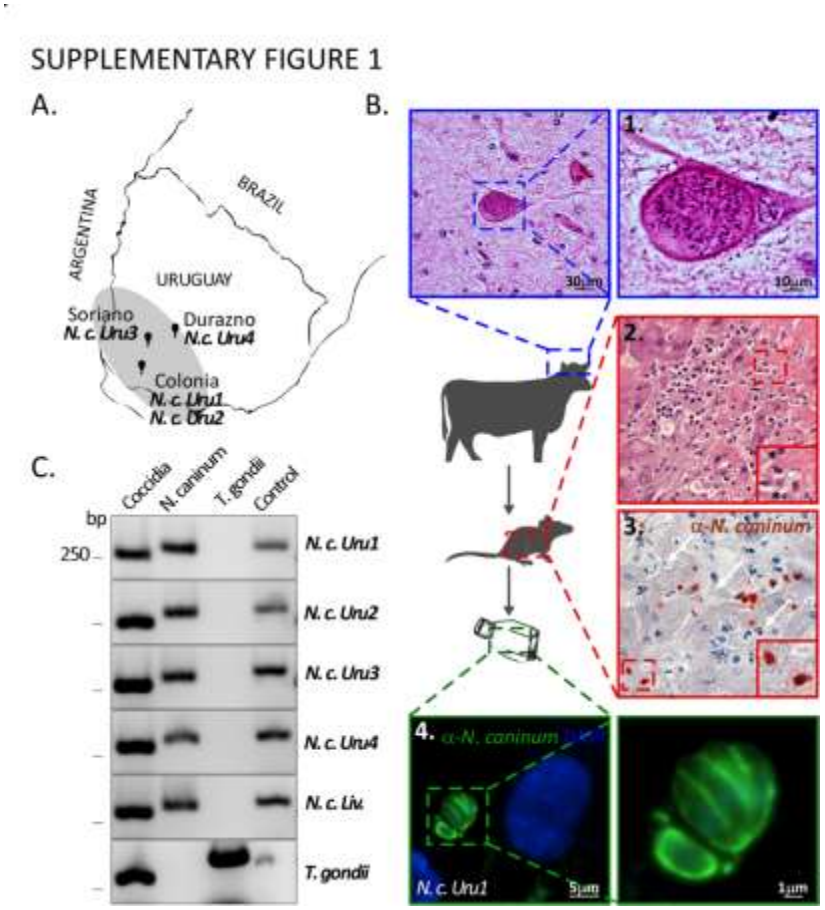

Figure S1. Four novel strains of *N. caninum* were isolated from congenitally infected calves. A. Schematic representation of Uruguay. **B. Schematic representation of the *N. caninum* strain isolation workflow.** Chronically infected pregnant cows, diagnosed by ELISA, were followed to term. Congenitally infected calves were euthanized, and PCR-positive brain sections subsequently inoculated into immunosuppressed (IFN□-KO) mice, from which strain isolation in tissue culture was attempted. **1b.** Representative histologic image of a protozoal tissue cyst found within the cytoplasm of a neuron in the brain of a calf infected with NcUru1. Hematoxylin and eosin stain. **2b.** Representative sections of a mouse´s liver infected with NcUru3, exhibiting *N. caninum* compatible lesions, which include infiltration of inflammatory cells (asterisk, inset) and hepatocellular necrosis (white arrowhead, inset). Hematoxylin

and eosin stain. **3b**. The region of inflammatory cell infiltration and hepatocellular necrosis colocalizes with multiple spots of chromogen deposition (immunoreactivity) with size and shape resembling tachyzoites, specifically-labeled with an anti-*N. caninum* antibody; two of these structures are shown in inset for clarity. Immunohistochemistry for *N. caninum* with hematoxylin counterstain. **4b**. Immunofluorescence assay using an anti-*N. caninum* antibody labels NcUru1 in *in vitro* cell culture. **C. Polymerase chain reaction confirms the isolates´ identity.** DNA extracted from the samples indicated on the right were subjected to PCR using primers with the specificities indicated above. *N. c. Liverpool* and *T. gondii* DNA were used as specific positive and negative controls for the primer pairs indicated.

SUPPLEMENTARY FIGURE 2

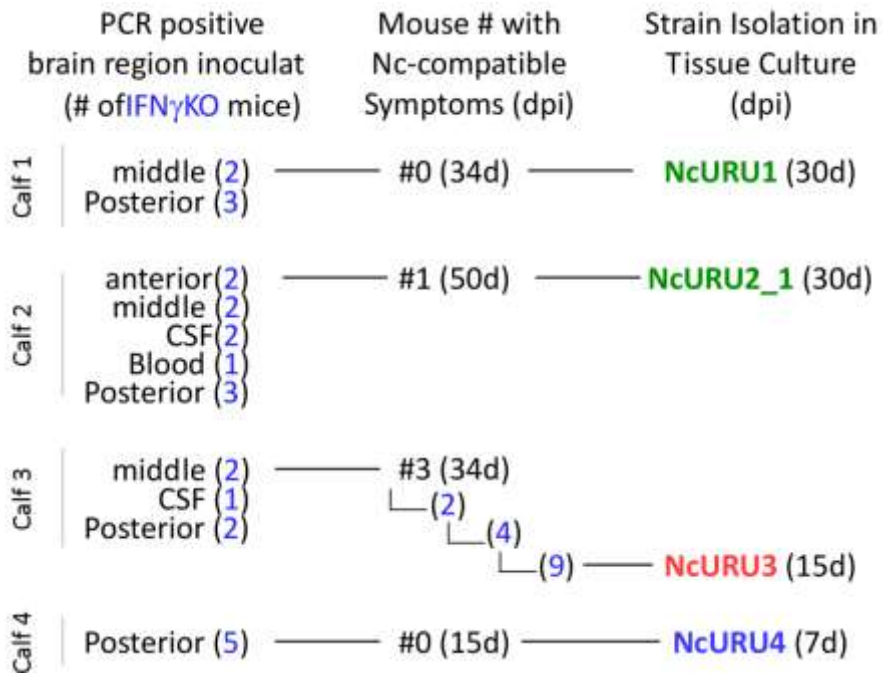

Figure S2. Isolation Scheme of *N. caninum* strains NcUru1-4. Ten calves were euthanized to attempt isolation. Of these, four resulted in successful isolation of N. caninum in tissue culture. Different brain sections (stem, middle, posterior, or anterior) and/or fluids (blood or cerebrospinal fluid - CSF) were analyzed, as indicated, by PCR. N. caninum-positive regions were inoculated into the number of IFN-□-KO mice indicated between brackets. Mice with neosporosis-compatible clinical signs were humanly euthanized at the times indicated, and their brains tested by PCR. PCR-positive brains were inoculated onto human foreskin fibroblast. Tachyzoites- compatible morphologies were first observed at the times indicated between brackets. Species identity was confirmed by PCR.

SUPPLEMENTARY FIGURE 3

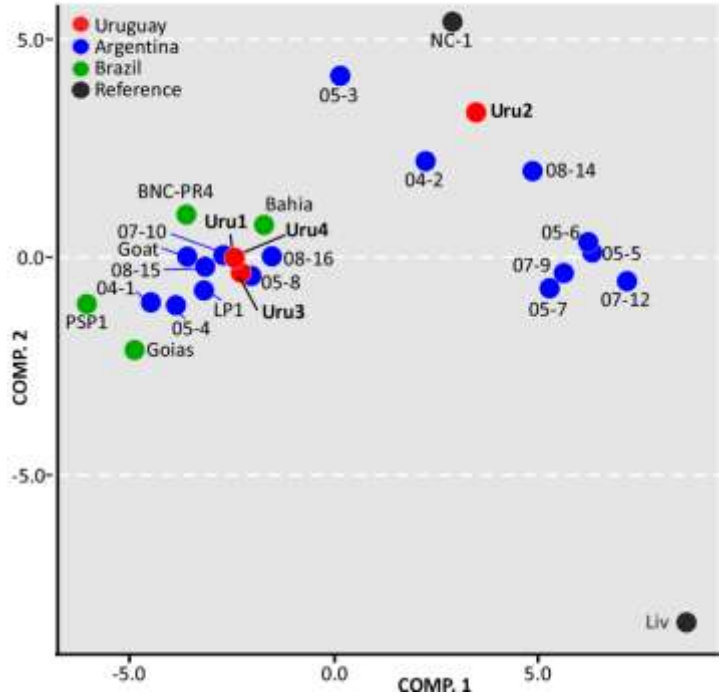

Figure S3. Clustering of regional *N. caninum* strains based on a principal component analysis (PCA).

SUPPLEMENTARY FIGURE 4

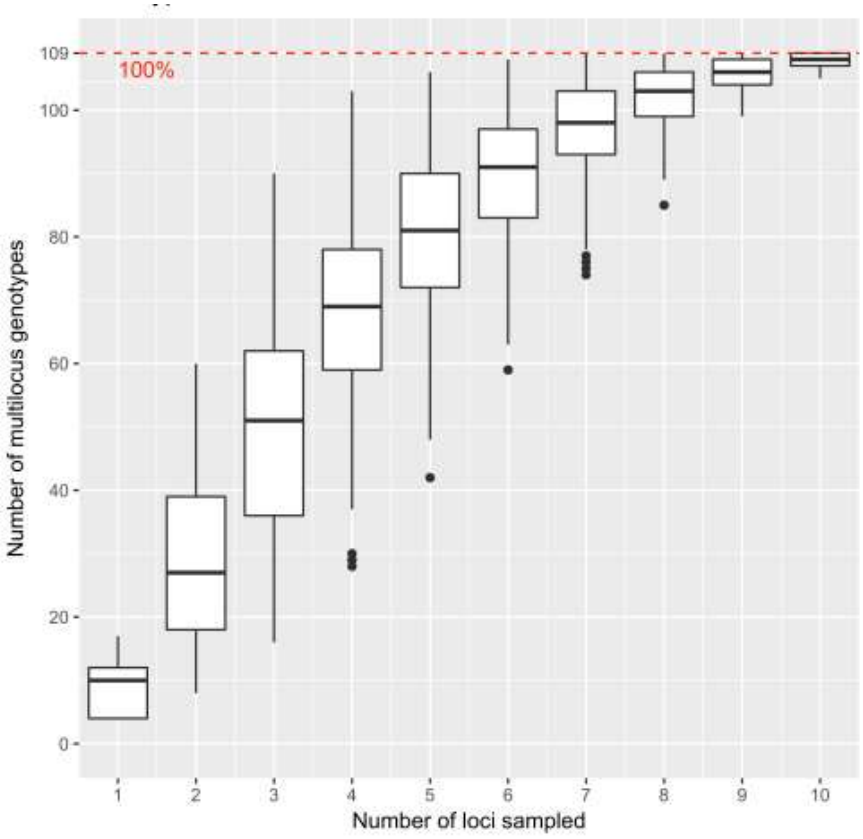

Figure S4. Genotype Accumulation curve for microsatellites

SUPPLEMENTARY FIGURE 5

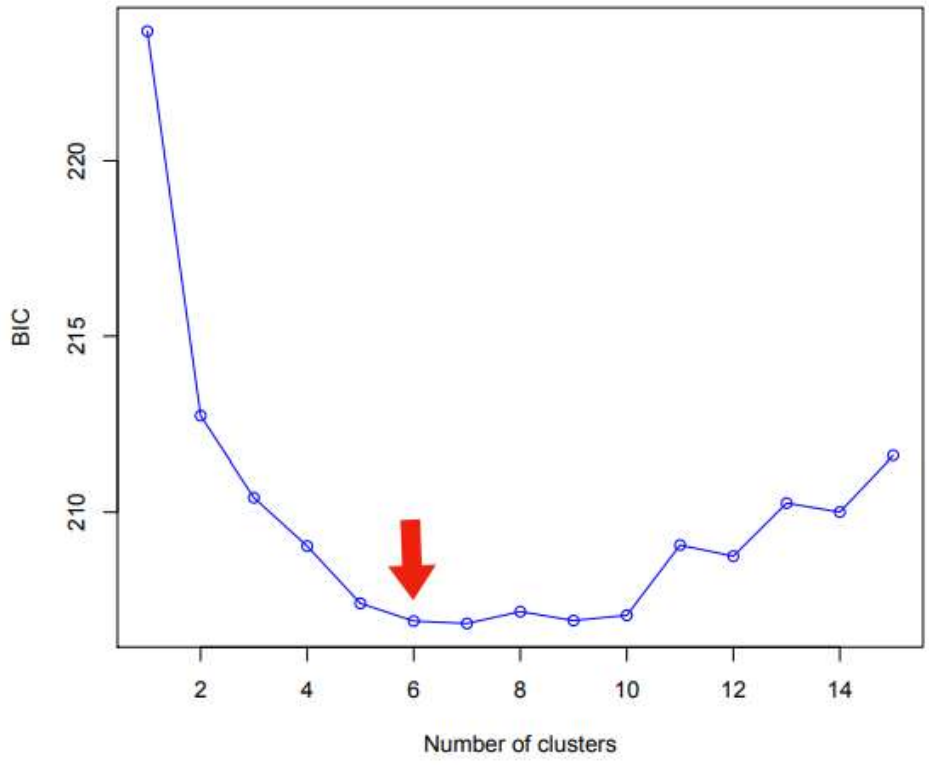

Figure S5. Number of statistically supported individual genetic clusters

SUPPLEMENTARY TABLE 1

Geographical Origin	Identifier_Host	Name	Sample (Abortion strain/ Isolated strain)	cluster	Reference
Argentina	AM_Argentina_goat	ARG-goat	A	1	**Campero, 2018**
	AM_Argentina_cow	ARG-04-1			**Regidor-Cerrillo, 2013**
	AM_Argentina_cow	ARG-05-4			
	AM_Argentina_cow	ARG-05-8			
	AM_Argentina_cow	ARG-07-10			
	AM_Argentina_cow	ARG-08-14			
	AM_Argentina_cow	ARG-08-16			
Brazil	AM_Brazil_cow	BNC-PR4	I		**Locatelli, 2018**
Spain	EUc_Spain_cow	SP-05-GAL-32	A		**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-05-GAL-35			
	EUc_Spain_cow	SP-05-GAL-36			
	EUc_Spain_cow	SP-05-GAL-38			
	EUc_Spain_cow	SP-05-GAL-42			
	EUc_Spain_cow	SP-07-GAL-49			
Iran	AS_Iran_cow	Nc-Iran2	A		**Salehi, 2015**
Mexico	AM_Mexico_cow	Mex-10-10	A		**Medina-Esparza, 2016**
Uruguay	AM_Uruguay_cow	**URU-1**	**I**		This study
	AM_Uruguay_cow	**URU-3**	**I**		
	AM_Uruguay_cow	**URU-4**	**I**		
Brazil	AM_Brazil_dog	Nc-Bahia	I	2	**Regidor-Cerrillo, 2006**
	AM_Brazil_cow	Nc-Goias	I		**García-Melo, 2009**
	AM_Brazil_cow	Nc-Goias1			**Oliveira, 2017**
	AM_Brazil_cow	Nc-PSP1			
Spain	EUc_Spain_cow	SP-03-ZAR-NcSp2H	I		**Regidor-Cerrillo, 2008**
	EUc_Spain_cow	SP-03-NAV-NcSp3H	I		
	EUc_Spain_cow	SP-03-NAV-NcSp4H	I		
	EUc_Spain_cow	SP-03-MAD-2	A		**Pedraza-Díaz, 2009**
	EUc_Spain_cow	SP-05-MAD-6			
	EUc_Spain_cow	SP-03-ZAR-7			

	EUc_Spain_sheep	SP-10-JAEN-8			**Moreno, 2012**
	EUc_Spain_cow	SP-01-GAL-14			**Pedraza-Díaz, 2009**
	EUc_Spain_cow	SP-01-GAL-15			
	EUc_Spain_cow	SP-00-GAL-17			
	EUc_Spain_cow	SP-01-GAL-18			
	EUc_Spain_cow	SP-02-GAL-20			
	EUc_Spain_cow	SP-01-GAL-21			
	EUc_Spain_cow	SP-01-GAL-23			
	EUc_Spain_cow	SP-02-GAL-24			
	EUc_Spain_cow	SP-02-GAL-25			
	EUc_Spain_cow	SP-02-GAL-26			
	EUc_Spain_cow	SP-04-GAL-27			**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-05-GAL-34			
	EUc_Spain_cow	SP-05-GAL-39			
	EUc_Spain_cow	SP-05-GAL-40			
	EUc_Spain_cow	SP-06-GAL-47			
	EUc_Spain_cow	SP-06-GAL-48			
	EUc_Spain_cow	SP-07-GAL-50			
Germany	EUc_Germany_cow	GER-08-2	A		
	EUc_Germany_cow	GER-08-3			
Mexico	AM_Mexico_cow	Mex-11-1	A		**Medina-Esparza, 2017**
Scotland	EUi_Scotland_cow	SCOT-08-3	A		**Regidor-Cerrillo, 2013**
China	AS_China_cow	Nc-LY	I		**Qian, 2017**
Spain	EUc_Spain_cow	SP-01-GAL-19	A		**Pedraza-Díaz, 2009**
	EUc_Spain_cow	SP-05-GAL-33			**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-05-GAL-41			
	EUc_Spain_cow	SP-06-GAL-46			
	EUc_Spain_cow	SP-07-GAL-53			
Germany	EUc_Germany_cow	GER-05-7	A	**3**	
	EUc_Germany_cow	GER-09-9			
Japan	AS_Japan_sheep	Nc-Sheep	A		**Regidor-Cerrillo, 2006**
Mexico	AM_Mexico_cow	Mex-11-2	A		**Medina-Esparza, 2017**
	AM_Mexico_cow	Mex-10-13			
	AM_Mexico_cow	Mex-12-21			
	AM_Mexico_cow	Mex-12-22			
Uruguay	AM_Uruguay_cow	**URU-2**	**I**		This study
Argentina	AM_Argentina_cow	ARG-04-2	A	**4**	**Regidor-Cerrillo, 2013**
Spain	EUc_Spain_cow	SP-03-LEON-NcSp5H	I		**Regidor-Cerrillo, 2008**

	EUc_Spain_cow	SP-05-GAL-31	A		**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-06-GAL-43			
Germany	EUc_Germany_dog	Hh-Berlin			**Regidor-Cerrillo, 2006**
	EUc_Germany_cow	GER-08-4			**Regidor-Cerrillo, 2013**
	EUc_Germany_cow	GER-05-6			
	EUi_Scotland_cow	SCOT-08-4			
Scotland	EUi_Scotland_cow	SCOT-08-5			
	EUi_Scotland_cow	SCOT-08-9			
Argentina	AM_Argentina_cow	ARG-08-15	A	**5**	
China	AS_China_dog	NC-LYDog	I		**Qian, 2016**
Spain	EUc_Spain_cow	SP-03-VAS-NcSp6	I		**Regidor-Cerrillo, 2008**
	EUc_Spain_cow	SP-05-MAD-1	A		
	EUc_Spain_cow	SP-03-MAD-3			**Pedraza-Díaz, 2009**
	EUc_Spain_cow	SP-05-MAD-5			
	EUc_Spain_cow	SP-08-MAD-9			**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-08-MAD-10			
	EUc_Spain_cow	SP-09-MAD-12			
	EUc_Spain_cow	SP-01-GAL-16			**Pedraza-Díaz, 2009**
	EUc_Spain_cow	SP-01-GAL-22			
	EUc_Spain_cow	SP-04-GAL-28			**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-04-GAL-30			
	EUc_Spain_cow	SP-06-GAL-44			
	EUc_Spain_cow	SP-06-GAL-45			
	EUc_Spain_cow	SP-07-GAL-51			
Germany	EUc_Germany_dog	Nc-GER1	A		**Regidor-Cerrillo, 2006**
Korea	AS_Korea_cow	KBA1	A		
Scotland	EUi_Scotland_cow	SCOT-07-1	A		**Regidor-Cerrillo, 2013**
USA	AM_USA_dog	NC-1	I		
Argentina	AM_Argentina_cow	ARG-05-3	A	**6**	
	AM_Argentina_cow	ARG-05-5			
	AM_Argentina_cow	ARG-05-6			
	AM_Argentina_cow	ARG-05-7			
	AM_Argentina_cow	ARG-07-9			
	AM_Argentina_cow	ARG-07-12			
China	AS_China_cow	Nc-LY2	A		**Qian, 2017**
Spain	EUc_Spain_cow	SP-03-MAD-NcSp1H	I		**Regidor-Cerrillo, 2008**
	EUc_Spain_cow	SP-03-NAV-NcSp7			
	EUc_Spain_cow	SP-03-NAV-NcSp8			

	EUc_Spain_cow	SP-03-NAV-NcSp9			
	EUc_Spain_cow	SP-06-MAD-NcSp10			
	EUc_Spain_cow	SP-05-GAL-37	A		**Regidor-Cerrillo, 2013**
	EUc_Spain_cow	SP-07-GAL-52			
Great Britain	EUi_GreatBritain_dog	Nc-Liv	I		**Regidor-Cerrillo, 2006**
Germany	EUc_Germany_cow	GER-00-1	A		**Regidor-Cerrillo, 2013**
	EUc_Germany_cow	GER-09-5			
	EUc_Germany_cow	GER-09-8			
Iran	AS_Iran_cow	Nc-Iran1	A		**Salehi, 2015**
Italy	EUc_Italy_cow	Nc-PV1	A		**Regidor-Cerrillo, 2006**
Korea	AS_Korea_cow	KBA2	A		
Scotland	EUi_Scotland_cow	SCOT-08-2	A		**Regidor-Cerrillo, 2013**
	EUi_Scotland_cow	SCOT-08-6			
Sweden	EUc_Sweden_cow	Nc-SweB	A		**Regidor-Cerrillo, 2006**

Distribution of *N. caninum* isolated strains or typed samples from abortions into genetic groups as determined by discriminant analysis of principal components (Graphically shown in Figure 1)

SUPPLEMENTARY TABLE 2

Genetic Group	1	2	3	4	5	6
Sample	Probability of belonging to a given group					
#1	**0.999**	0.000	0.000	0.001	0.000	0.000
#2	**0.358**	**0.642**	0.000	0.000	0.000	0.000
#3	**0.715**	0.000	**0.248**	0.000	0.037	0.000
#4	**0.742**	0.057	**0.158**	0.000	0.036	0.008
#5	**1.000**	0.000	0.000	0.000	0.000	0.000
#6	0.000	0.000	0.028	0.000	**0.971**	0.001
#7	**0.973**	0.007	0.001	0.000	0.017	0.001
#8	0.001	**0.488**	0.000	0.000	0.001	0.510
#9	0.000	0.000	**0.650**	0.000	0.045	**0.305**
#10	0.002	**0.998**	0.000	0.000	0.000	0.000
#11	0.029	**0.971**	0.000	0.000	0.000	0.000
#12	0.003	**0.997**	0.000	0.000	0.000	0.000
#13	0.000	0.000	**0.707**	0.000	0.003	**0.290**
#14	**0.589**	0.050	0.018	0.000	**0.139**	**0.203**
#15	0.000	0.000	**0.316**	0.000	**0.592**	0.091

Distribution of *N. caninum* typed samples from abortions into each genetic group as determined by a discriminant analysis of principal components (Graphically shown in Figure 3). Probabilities greater than 0.1 have been highlighted.

4.0 Diferentes grados de diversidad genética y fenotípicas en cuatro aislados nacionales.

El éxito en el aislamiento de cuatro nuevas cepas de *N. caninum* de diferentes regiones del país (Colonia, Soriano y Durazno) y con antecedentes diferentes (obtenidos a partir de una tormenta de aborto o de terneros congénitamente infectados y asintomáticos), generó un insumo de gran importancia. Por un lado, el estudio de marcadores genéticos permitió comparar entre sí a la cepas uruguayas, resaltando la existencia de una amplia variabilidad genética. Por otro lado, observamos que dos cepas con antecedentes dispares (transmisión vertical / trasmisión horizontal con tormentas de abortos) tenían el mismo conjunto de marcadores genéticos.

A la variabilidad genética observada utilizando los marcadores genéticos, se le suma la observación de comportamientos diferentes *"in vitro"* e *"in vivo"* que notamos mientras realizamos sus respectivos aislamientos. Por ejemplo, veíamos que algunas cepas crecían más rápidamente en los cultivos celulares que otras. Así como también, generaban más o menos tempranamente sintomatología y muerte en el modelo murino.

Este conjunto de variantes nos llevó a plantearnos diferentes interrogantes. Nos preguntamos; ¿Qué fenotipos característicos tienen nuestras cepas autóctonas? ¿Qué diferencias tienen con la cepa virulenta de referencia? ¿Pueden estas diferencias explicarse por diferencias en el crecimiento e invasión de estas nuevas cepas en un modelo *"in vitro"*? ¿Cómo impactan estos fenotipos en la transmisión vertical en un modelo murino? Y finalmente, ¿Cuáles son las bases moleculares de estas diferencias?

La caracterización fenotípica de estas nuevas cuatro cepas tanto en los modelos “in vitro” como en el *“in vivo”* nos planteó un nuevo desafío.

Este desafío nos llevó a optimizar el modelo *“in vitro”* para analizar los fenómenos que llevan a establecer una infección exitosa. Los ensayos de comportamiento en cultivo celular nos permiten medir, por ejemplo, la capacidad infectiva y de proliferación cepa-específicos.

Paralelamente se desarrolló el modelo *“in vivo”* que nos permitió realizar ensayos de virulencia comparando nuestras cepas con la cepa de referencia NcLiverpool. Sumado a los modelos de virulencia, se logró establecer el modelo de transmisión vertical. Este último, nos permite evaluar la capacidad de transmisión del parásito a la progenie y como esta transmisión afecta su viabilidad, visualizando la potencialidad de cada cepa de generar abortos.

Con el objetivo de profundizar en la base molecular de las diferencias fenotípica, secuenciamos los diferentes genomas ya que está bien establecido en la literatura (y en nuestro caso esto se hace explícito en el caso de NcUru1 y NcUru4) que el uso de microsatélites como marcadores genéticos no logra resolver las diferencias genéticas que expliquen las diferencias fenotípicas.

Los datos de este estudio nos permitieron establecer que nuestras cepas aisladas son de baja virulencia y con características singulares a nivel fenotípicos. Así mismo, la secuenciación de sus genomas es insumo que a futuro nos permitirá profundizar en las singularidades genéticas que subyacen a estos fenotipos. Abriendo la puerta por ejemplo, para la formulación de una posible vacuna modificando cepas antigénicamente relevantes para el país (por ejemplo, cepas circulantes). Basados en el conocimiento de las variantes genéticas que generan cepas de baja virulencia, para modificarlas de manera dirigida.

Los resultados de estos estudios se resumen a continuación en el manuscrito que será enviado a una revista científica en simultáneo a la evaluación de esta tesis.

4.1 Four Neospora caninum isolates from adjacent regions exhibit high degree of genetic and phenotypic diversity

Authors and affiliations: **Andres Cabrera[1,2], Luisa Berná[1], Lucía Lopez[1] , Ana Paula Arevalo[3], Martina Crispo[3], Maria E. Francia[4,5], Carlos Robello[1, 6]**

1 Laboratory of Host Pathogen Interactions-UBM. Institut Pasteur de Montevideo. Montevideo, Uruguay.

2 Unidad de Microbiología, Departamento de Patobiología. Facultad de Veterinaria-Universidad de la República. Montevideo, Uruguay.

3 Transgenic and Experimental Animal Unit. Institut Pasteur de Montevideo. Montevideo, Uruguay

4 Laboratory of Apicomplexan Biology. Institut Pasteur de Montevideo. Montevideo,

5 Departamento de Parasitología y Micología. Facultad de Medicina-Universidad de la República. Montevideo, Uruguay.

Uruguay.

6 Departamento de Bioquímica. Facultad de Medicina-Universidad de la República. Montevideo, Uruguay.

Keywords *Neospora caninum*, Mouse model, Vertical transmission, Animal Health

Running title: *N. caninum* Genetic and phenotypic variability

Introduction

Neospora caninum is an obligate intracellular coccidian parasite that causes neosporosis. It actively invades cells within its hosts, and persists chronically in encysted forms which can reactivate during pregnancy and be efficiently transmitted to the fetus. Bovine neosporosis is one of the leading causes of bovine abortion in the world and it is known to cause of billions dollars in losses (Reichel et al., 2013).

Drugs to efficiently treat the chronic form of the disease are unavailable. Vaccination is considered the best potential control strategy against bovine neosporosis, as it offers the best cost benefit ratio (Reichel and Ellis, 2006). Several strategies for the formulation of a vaccine against *N. caninum* have been proposed, from whole parasite lysates to recombinant antigens, and even live attenuated parasites (Hecker et al., 2014; Jenkins et al., 2004; Mazuz et al., 2015; Nishimura et al., 2013; O'Handley et al., 2003; Rojo-Montejo et al., 2013; Romero et al., 2004). It is currently accepted that the latter represents the most promising short-term option for the control of neosporosis (Reichel et al., 2015). The isolation of attenuated parasite strains, and the identification of the molecular underpinnings of such phenotypes is paramount for the optimization of vaccine formulations. In this regard, molecular studies over the past 10 years have documented the occurrence of phenotypic variability in different parasite isolated worldwide, on the backdrop of ample genetic variability in the form of microsatellites and single nuclear polymorphisms (Cabrera et al., 2019; Chryssafidis et al., 2014; Khan et al., 2019; Rojo-Montejo et al., 2009b). However, the correlation between genetic and the phenotypic variability is still unclear. Further characterization of strains is vital to further understand the biology of the parasite.

In previous work, we isolated four strains of *N. caninum*, from different regions of Uruguay (Cabrera et al., 2019). We determined these four strains to represent three distinct genetic lineages

based on microsatellite typing, differing significantly from other extensively characterized isolates, such as the reference strain N. caninum Liverpool (NcLiv). We also observed that some lineages were more closely related to abortion causing strains than others foretelling possible phenotypical differences amongst isolates. Microsatellite configurations are known, however, not to correlate with phenotypic traits. This is illustrated herein by the fact that NcUru1 and NcUru4 are identical at the microsatellite level, although they arose from farms with very different histories in terms of abortions, whereby NcUru1-3 arose from endemic herds with abortions caused by Neosporosis, being rare while NcUru4 originated from a herd that underwent an acute infection and abortion storm.

In this study we set out to characterize the phenotypes of isolates NcUru1 thru 4, and their correlation with genetic variability. We identify marked differences amongst these N. caninum strains in terms of virulence and their impact on reproductive fitness, in the mouse model. We put forward a potential vaccine-candidate strain which not only protects mice from developing neosporosis but also provides protection against chronic infection, abortion and vertical transmission, in a pregnant mouse model. Furthermore, we investigate the basis of these differences, and identify mechanistic links with differences in invasion and intracellular replication efficiency, as well as, molecular clues in the form of single nuclear polymorphisms by whole genome sequencing.

Material and methods

Cell culture and Balb/c mice inoculation.

N.c. Liverpool was acquired from ATCC (Cat.#: 50845). N.c.Uru1-4 were isolated from local congenitally infected calves, cultured and maintained by serial passages as described in Cabrera et al. 2019. Tachyzoite numbers were determined by counting syringe lysed individual parasites, filtered through a 3µm filter, in a Neubauer chamber. For eliciting acute neosporosis, an LD_{50} of 10^7 NcLiv tachyzoites injected intraperitoneally was empirically determined in Balb/c female mice of 6-8 weeks of age. This dose was used in virulence assays. Chronic disease was induced by intraperitoneal inoculation of 10^6 tachyzoites in Balb/c female mice of 6-8 weeks of age. This dose was used for vertical transmission assays. All strains used in phenotypic and *in vitro* assays were passaged less than 15 times from the time of isolation.

Invasion and intracellular growth assays

For the intracellular growth assay, confluent monolayers of Human foreskin fibroblasts (HFFs) were grown on coverslips, and subsequently inoculated with 10^3 freshly released tachyzoites. Two hours post-inoculation cells were washed five times with PBS to remove unattached parasites, and fixed with 4% paraformaldehyde 24h later. Samples were analyzed by immunofluorescence assays, as described in Cabrera, et. al, 2019. The number of parasites per vacuole in 200 vacuoles per coverslip was determined, in triplicate. For invasion assays, 1000 parasites were inoculated onto coverslips containing confluent HFFs, kept on ice prior to the assay. Upon inoculation with parasites, plates were centrifuged at 300 x g for 10 minutes at 4ºC to ensure all strains contacted the cells simultaneously. Plates were then switched to 37ºC for 30 minutes, washed with PBS three times to remove unattached parasites, and fixed with 4%

paraformaldehyde. The number of parasites per field, in 50 fields was determined for all strains, in triplicate by immunofluorescence assay as described above.

***In vivo* studies.**

For "*in vivo"* studies, six-week-old female BALB/c mice were used following approved animal protocols. Animals were maintained in a controlled environment with 12-h light/dark cycles with feed and water *ad libitum*.

For virulence studies, mice were housed in groups of five. Mice were intraperitoneally injected with one parasite strain at time, at a dose of 10^6 resuspended in 250 μL of sterile PBS. The uninfected control group was injected with the same volume of sterile PBS. Mice were examined daily for 30 days post inoculation to detect clinical signs compatible with neosporosis (rough fur, inactivity, anorexia and neurological signs). Seropositivity against *N. caninum* infection was systematically tested two weeks post-infection. Sera were tested by immunoblotting against *N. caninum* tachyzoites lysates. Symptomatic mice were humanely euthanized and brain samples were aseptically obtained to identify *N. caninum* by PCR. For protection studies, seropositive mice who did not develop clinical symptoms, were re-challenged with the LD_{50} of *N.c.* Liverpool, and followed along, as described above.

For vertical transmission studies, female mice were housed in groups of six. Mice were intraperitoneally injected with a dose of 10^6 tachyzoites of different strains to induce chronic disease. The control group was injected with sterile PBS solution (uninfected animals). At 8-10 weeks of age, mice were synchronized for one week by housing them with male bedding. Three females were placed with a single male for seven nights. The presence of a vaginal plug was corroborated in all cases as a positive sign of mating. Day three of females housing with males was considered day 0 of pregnancy. Pregnant mice and fetuses were sacrificed at day 20 post-

pregnancy. Brains of adult mice, reabsorbed fetuses, viable and aborted fetuses were collected for molecular assays. Sera was collected for serological assays.

Sequencing, alignment and variant calling

DNA was extracted from *N. caninum* NcUru1, NcUru2 and NcUru3 strains using a DNA purification kit from Zymo Research (#D4074). Illumina libraries were performed as previously described (Berná et al., 2018) and whole genome sequencing of the three strains was performed by Integrative Genomics Core to 75x coverage with 2x100 paired-end cartridges. Paired reads were mapped to the new reference *N. caninum* Liverpool genome (https://doi.org/10.1101/2020.05.22.111195) using Burrows-Wheeler Aligner (BWA 0.7.17) (Li et al., 2009) mem using default parameters. Samtools v1.9 was used to post process alignment files (Li et al., 2009). SNP and Indel discovery was performed with Samtools mpileup (Danecek et al., 2011) and VarScan. v2.3.9 (Koboldt et al., 2012) with parameters --min-reads2 3 --min-coverage 3 --min-var-freq 0.8. SNPeff v.5.0 was used to annotate genomic variants and predict its functional effects. IGV was used for alignment and variants visualization (Robinson et al. 2011). Venny 2.1 was used for venn diagram plots (Oliveros, 2007)

Ethics statement.

All experiments involving animals were done following pre-approved protocols by Institutional ethic committees (CEUA # 010-17) at the Institut Pasteur de Montevideo.

Results

To comparatively study the phenotypes of NcUru 1-4 we first evaluated their virulence "*in vivo"*. To do this, we infected immunocompetent mice with 10^7 freshly parasites of each of the four isolates, or with NcLiv as positive control. An equivalent volume of PBS was used as a negative control. Parasite viability was assessed in parallel by plaque assay. Mice seroconversion was controlled two weeks post-inoculation. Clinical signs of the disease (anorexia, rough coat, and inactivity followed by nervous signs like rounded back, pelvic limb weakness and walking in circles) were monitored for a month post inoculation.

Mice infected with NcUru1-4 showed no obvious clinical signs of the disease, and exhibited the same pattern curve than control, i.e. 100% survival, whereas the 60% of the mice infected with the reference strain NcLiv died (Figure 1). To further corroborate proper parasite inoculation, and to approximate each strain's ability to switch from an acute to a chronic infection we evaluated the presence of each strain in the mice's brains. Though all strains were detectable by PCR in the mice's brains, there were marked differences: whereas100% of mice infected with NcLiv were positive individuals whereas NcUru1-4 were detected in 30%, 50%, 60% and 40% of individuals respectively, suggesting that they differ in their ability not only to cause clinical signs of the disease, but also to persist in the host (Fig 1).

Next, we assessed the relative impact of each strain on pregnancy outcomes. To do this, we optimized a vertical transmission model. We infected six-week old Balb/c female mice with 10^6 parasites in order to generate subclinical chronic infection. We next crossed females and males, whereby subgroups of females infected with the same parasite strain were crossed with the same male. Chronic infection of the females was corroborated postmortem by PCR of the brains once the assay was finalized. Pregnancy rate, abortion rate and the rate of vertical transmission for

each strain were evaluated. Pregnancy rates varied from 28% for females inoculated with NcLiv (positive control), to 48% for the control group (PBS) (Fig 2A). The Uruguayan isolates exhibited strain-specific variability within the ranges observed for the positive and negative controls. The rate at which pregnancy was established in females infected with NcUru1 was comparable to that of NcLiv (28%), NcUru4 behaved similarly to the negative control (PBS- 48%), NcUru2 and NcUru3 presented pregnancy rates of 36% and 37%, respectively (Fig 2).

We further examined the frequency of progeny viability by performing a c-section a day before the expected due date. We observed that virtually all offsprings were viable in both negative and positive control groups; 98% and 100%for PBS and NcLiv, respectively (Fig 2B). These figures did not vary regardless of whether the pregnant mouse was later determined to either be effectively infected chronically or non-infected. On the other hand, when we evaluated the progeny of mice infected with our isolates, we observed that the output of viable offspring in the groups of chronically infected mothers significantly decreases. Mothers infected with NcUru2, bear only 63% of the viable offspring, and NcUru4, NcUru3 and NcUru1 infected mothers yielded, 75%, 77%, and 81% viable offspring. Of note, NcUru1 infected mice produced viable offspring at a rate comparable to that of the control and reference groups (Fig 2B).

We next evaluated offspring viability in mice who notwithstanding having been inoculated with a given strain, has undetectable *N. caninum* DNA in their brain (i.e. regarded in this study as not having established a chronic infection). We did not obtain data for the NcUru1 and NcUru4 infected groups because none of the non-chronically infected mice got pregnant. NcUru2 inoculated mice had 100% viable offspring. In contrast, new progeny viability for the NcUru3 non-chronically infected mothers was halved in (50%) (Fig 2C). In order to compare strain-specific vertical transmission rates, we determined the percentage of *N. caninum*

PCR-positive fetuses' brains. Consistent with previous reports, we detected a vertical transmission rate for NcLiv of 87%. Vertical transmission rates for NcUru1 thru 4 were comparatively lower than that of NcLiv. NcUru2 was found in 20% of the progeny from mice infected with this strain, while the vertical transmission rate was of 29 and 17% for progeny of mice infected with, NcUru3 and NcUru4, respectively. Remarkably, NcUru1 transmitted to only 11% of the progeny.

It is well established for *N. caninum*'s close relative *Toxoplasma gondii*, that the capacity to invade cells and velocity of intracellular growth correlates with virulence (Murillo-León et al. 2019). In order to approach the molecular basis of the phenotypic differences observed *in vivo* among our *N. caninum strains*, we *in vitro* characterized each strain's ability to invade a host cell and their intracellular growth rate using NcLiv as a reference. Other than NcUru3, which invades host cells with an efficiency similar to that of the reference, NcUru1,NcUru2 and NcUru4 are comparatively less invasive (Fig 3A). NcUru1 is half as invasive as NcLiv, while NcUru2 invades roughly 25% less cells than NcLiv. Remarkably, NcUru4 presents an extremely low invasion efficacy (18%) (Fig 3A). Conversely, when we evaluated the relative growth of each strain compared to the NcLiv, we found that NcUru4 is the only strain exhibiting a similar growth rate to that of NcLiv, suggesting that those parasites which manage to invade in the assay's allotted time, are equally able to grow intracellularly as is the reference. NcUru1 (72%), NcUru2 (53%) and NcUru3 (38%) were comparatively slower growing than NcLiv (Fig 3B).

The *in vivo* and *in vitro* evaluation of NcUru1 thru 4 revealed not only marked differences between each and the reference strain, but also among the Uruguayan autochthonous strains themselves. Consistently, in previous work we determined that these strains were genotypically distinct. Specifically, NcLiv clustered in a markedly different genetic group from that of the uruguayan strains NcUru1, 3 and 4, whilst simultaneously, NcUru2 belongs to a distinct

cluster. Taking these results together, we hypothesized that there are likely differences at the genomic level amongst these strains underlying their specific phenotypic traits. In order to test this, we pursued whole genome sequencing for NcUru1-3 by Illumina. Unfortunately, we repeatedly failed to obtain sufficiently good quality DNA for sequencing NcUru4.

In previous work we re-sequenced and assembled the whole genome of NcLiv and NcUru1 using third generation sequencing, and showed that the *N. caninum* genome previously accepted as the reference, had been assembled incorrectly. Furthermore, we demonstrated that the genomes of NcLiv and NcUru1 are virtually identical regarding gene composition, genome organization and synteny conservation. No large rearrangements or deletions were detected. Using our newly assembled NcLiv genome as the reference, we mapped the illumina sequences of NcUru1-3, and identified their respective differences. A total of 2591 different variants (SNPs and Indels) were discovered among NcUru1-3 and NcLiv . Of these, 1819 are located in coding regions. nly 143 mutations are common to the three Uruguayan strains while the majority of these are strain-specific (189 in NcUru1, 721 in NcUru2 and 455 in NcUru3). Strikingly, we do not find in our data previously reported "hotspot" of single nucleotide polymorphisms amongst world-wide isolates (Khan, et al., 2019). The genes affected by strain-specific SNPs are mostly annotated as mediating protein-protein interactions, transcription and translation, protein binding, ribosomal subunit formation, kinase activity, and protein phosphorylation:Ribosomal protein L6e (NCLIV_056680) ribosomal protein S6 (NCLIV_062450) PAD-1 ubiquitin protease (NCLIV_037150) ribosome biogenesis protein BMS1(NCLIV_057070) proteasome (Prosome, macropain) subunit, beta type, 1AB1/Mov34/MPN/PAD-1 ubiquitin protease/Maintenance of mitochondrial structure and function(NCLIV_37150),inhibitor-1 of protein phosphatase type 2A (NCLIV_35310)cAMP-specific phosphodiesterase(NCLIV_103500) ALG6, ALG8

glycosyltransferase(NCLIV_25590) DEAD/DEAH box helicase/Helicase conserved C-terminal domain leucine carboxyl(NCLIV_43620) Leucine carboxyl methyltransferase(NCLIV51310) 4'-phosphopantetheinyl transferase(NCLIV_51620).

Discussion

In previous work, we isolated and genetically typed four *N. caninum* strains from Uruguay named NcUru1 thru 4(Cabrera et al., 2019). We further showed that while NcUru1, 3 and 4 grouped together genetically, they were markedly distinct from both NcUru2 and NcLiv, the latter being the strain commonly used as a reference for the study of *N. caninum*'s biology. Here, we characterized a number of strain-specific phenotypes relevant to the clinic of neosporosis using the mouse model as a proxy for intermediate host dynamics of infection. Even though the best model to phenotypically evaluate *N. caninum* is its natural host, the cow, only a handful of laboratories have carried out *in vivo* analysis on cows due to ethical considerations and allocation limitations (Horcajo et al., 2016). In spite of immunological differences between both models, the mousehas been used to model neosporosis, since the discovery of the parasite, mainly due to its reduced size, low cost, easy handling and short gestation period (Aguado-Martínez et al., 2017; López-Pérez et al., 2006). Through the use of the murine model, several strains of *N. caninum* have been phenotypically characterized, although attempts to correlate these phenotypes with the strain's genotype were not conclusive (Calarco et al., 2018).

The study of the Uruguayan strains' phenotypes revealed that they exhibit , in general terms, low virulence when compared to NcLiv; they fail to generate disease in the infected mice and in cases, they do not establish a chronic infection. These results are consistent with observations reported by others whereby low virulence strains do not elicit clinical disease or

establish chronic infection in mice (Regidor-Cerrillo et al., 2010). Nonetheless, subtle phenotypic differences were identified amongst all strains. Notably, NcUru1 exhibited a markedly reduced tendency to generate chronic infection. We observed that in generla, pregnancy rates were not influenced by the infection with *N. caninum*. The NcLiv vertical transmission rate (87%) observed in this work is comparable with that of previous studies confirming that this strain is very effectively transmitted by an infected mother (Quinn et al., 2002; Regidor-Cerrillo et al., 2010). The Uruguayan strains exhibited a very low vertical transmission rate compared to the reference strains, with NcUru1 being passed on to less than 1 every 5 progeny. Differences in vertical transmission rate have also been observed between another virulent isolate (Nc-1) and a number of low virulence strains isolated in Spain (Rojo-Montejo et al., 2009a). It is worth mentioning, however, that all of our isolates were obtained from congenitally infected calves, implying that they are indeed transmitted vertically in the field.

Whole genome sequencing of NcUru strains revealed that while most non-coding mutations are strain-specific, shared variants are present mostly in coding regions, suggesting that they are present in a common ancestor. A total of 143 mutations were identified in the three NcUru strains evidencing, albeit expected, differences with NcLIv that could be characteristic of regional strains. These variants could be related to phenotypic differences as they affect genes involved in the different pathways that could possibly modulate the host response. Previous studies demonstrated that the host's metabolism was highly regulated by the parasitic infection by *N. caninum*, directly affecting synthesis, turnover (ribosome and proteasome) and metabolism of carbon, glycolysis/gluconeogenesis, pentose phosphate pathway, amino sugar and cysteine and methionine.

Previous work using genome-wide SNPs on two NC strains shows multiple SNPs hotspot in different chromosomal regions (Calarco, 2020). A recent effort using seven additional Nc strains for whole-genome sequencing has evidenced hotspot common to five strains, with the majority of the SNPs being clustered in six regions (Khan, 2019). It should be noted however, that both these studies use the previous NcLiv reference genome, bearing missasemblies and wrongly defined chromosomes to map these hotspots. In addition, a large number of identified hotspot are not congruent on both experiments. Our results do not evidence clear new hotspots, nor do we detect SNPs in the aforementioned described hotspots. However, some chromosomal regions do not present enough coverage to detect hotspot variants in our experiment, due to low global coverage It could be possible that different strains present different hotspot patterns. In this regard, in the work by Khan & colleagues, two strains do not exhibit SNPs at the "hotspot"s, and conspicuously, these strains do not coincide with those suffering from low coverage sequencing (Khan, 2019).

In conclusion, we have shown that ample biological diversity exists between Uruguayan isolates. In particular, we note that these isolates are generally of low virulence. In the light of a correctly assembled and thoroughly annotated genome, we are currently working on deciphering the association of strain-specific mutations on different genes with their impact on virulence.

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Figure 1.

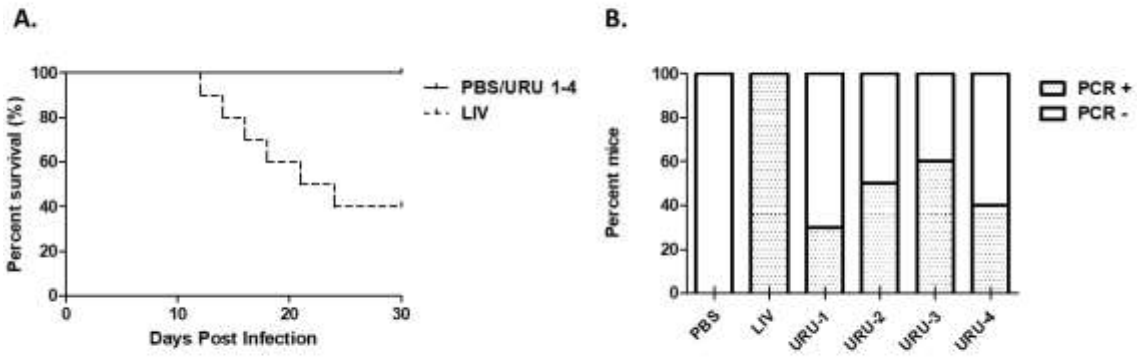


Figure 1. **The Uruguayan isolates exhibit comparatively lower virulence and tendency to evolve into a chronic infection than *NcLiv.*** **A**. Survival rate of parasite-infected mice inoculated with 10^7 tachyzoites of the indicated strain, or PBS (negative control) . **B**. Percentage of positive (+) or negative (-) detection of the parasite by PCR in the brains of mice inoculated with the indicated strains.

Figure 2.

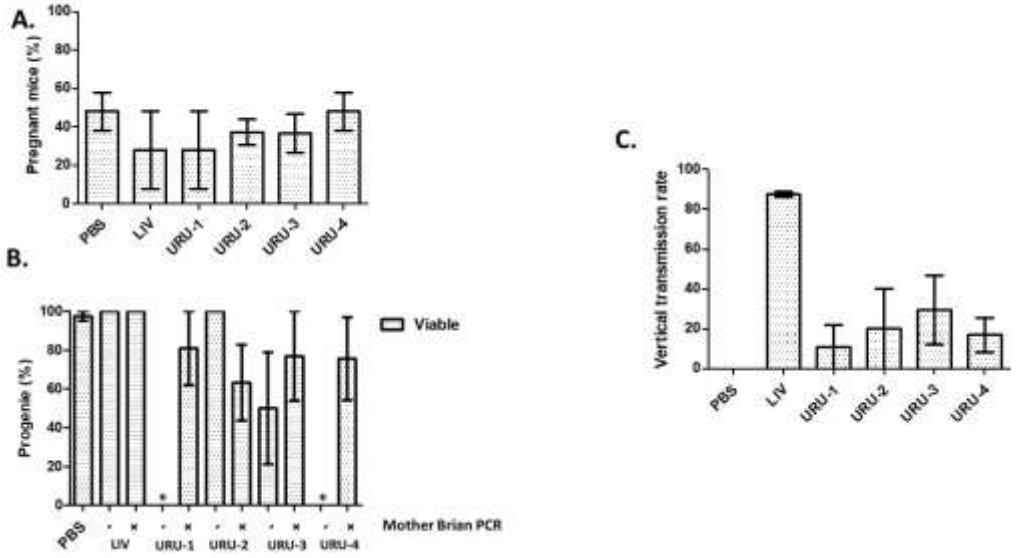


Figure 2.The Uruguayan isolates exhibit lower vertical transmission rates than the reference strain. A. Percentage of pregnant mice inoculated with the strains indicated. Data are shown as the mean and SEM of 3 independent experiments. **B.** Progeny Viability Percentage **C.** Vertical transmission rate.

Figure 3.

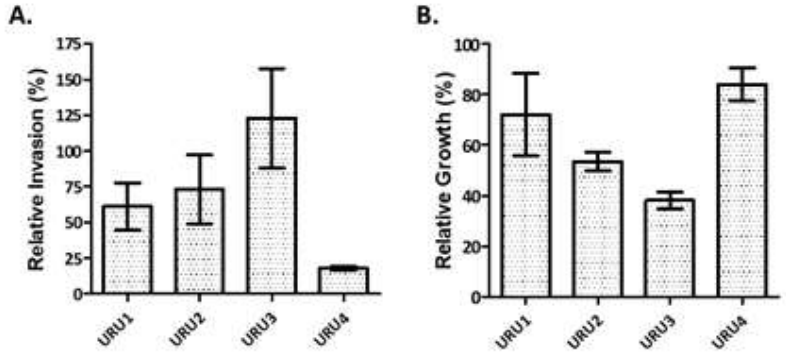


Figure 3. Invasion and intracellular growth of isolated strains were quantified and plotted in comparison to reference strain. A. Relative invasion rate of each strain was quantified against that of NcLiv (n=3, Average, SEM are plotted) **B.** Relative intracellular growth of each strain was quantified against that of NcLiv (n=3, Average, SEM are plotted).

Table 1. Statistics of whole genome sequencing of three *N. caninum* uruguayan strains

Strain	Aligned reads	Aligned reads (%)	Coverage (%)	Total SNPs	Total SNPs in coding genes
NcUru1	4.11E+06	9.5	6.8	638	531
NcUru2	8.19E+06	15.0	13.5	1436	1053
NcUru3	3.90E+06	6.1	6.4	1175	871

Figure 4.

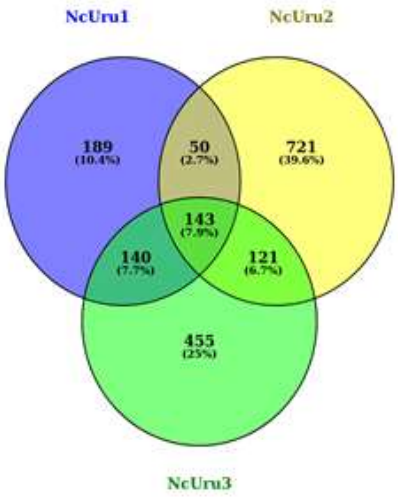


Figure 4. Venn diagram showing the SNPs comparing the reference strain with the Uruguayan low virulence isolates.

5.0 La reevaluación de los genomas de *Toxoplasma gondii y Neospora caninum* revela: ensamblaje incorrecto, diferencias de cariotipo y reordenamientos cromosómicos

La generación de una gran cantidad de datos a nivel genético y fenotípico de las cepas autóctonas y de referencia, y la dificultad de correlacionar los marcadores genéticos con estos fenotipos cepa-específicos nos llevó a buscar otras metodologías para abordar este problema.

El advenimiento de las nuevas tecnologías de secuenciación (PacBio, Oxford Nanopore e Ilumina), de la mano con la accesibilidad determinada por la baja de los precios, nos permitió utilizar estas potentes herramientas para responder nuevas preguntas sobre los datos ya generados de fenotipos con base en los genomas de los nuevos aislados. Algunas preguntas que nos planteamos fueron; ¿Cuáles son los marcadores/genes que pueden ser asociados a los diferentes fenotipos? ¿Qué diferencias encontramos en los genomas de las cepas aisladas y la de referencia? ¿Cuál es la estructura de sus genomas?

En el comienzo de esta tesis doctoral, el único genoma secuenciado en su totalidad y público en la base datos era el de la cepa de referencia Nc Liverpool. Este genoma fue secuenciado utilizando tecnologías de secuenciación de primera y segunda generación (Sanger), y ensamblado bajo la hipótesis de alta conservación de sintenia con *Toxoplasma gondii*, el cual fue utilizado como referencia.

En particular, las tecnologías de secuenciación de tercera generación, Pacific Bioscience (PacBio) y Oxford Nanopore superan a las tecnologías anteriores al proporcionar lecturas más

largas, que pueden abarcar regiones que contienen secuencias repetitivas, entre otras ventajas, permitiendo secuenciar completamente los nuevos genomas con un ensamblado de alta precisión.

La secuenciación de los nuevos genomas nos permitió identificar cambios en el paradigma de la composición cromosómica de *N. caninum* y de *T. gondii*. Observamos que los ensamblajes anteriores del genoma de *N. caninum*, basados en el genoma de *T. gondii*, eran incorrectos. Particularmente, detectamos grandes reordenamientos cromosómicos entre las especies. La secuenciación también permitió identificar nuevas regions del genoma con potencialidad de contener nuevas proteínas involucradas en factores de virulencia. Tal vez el hallazgo mas relevante de esta parte del trabajo fue que ambos genomas arrastraban un error de ensamblaje, producto de los fragmentos cortos generados por tecnologías de secuenciación anteriores. Los cromosomas ensamblados anteriormente como VIIa y VIII son de hecho un único cromosoma. Este hallazgo nos permitio corregir el cariotipo no solo de *N. caninum* sino también de *T. gondii*; ambas especies tienen 13 cromosomas y no 14.

Estos resultados, generados por la Dra. Luisa Berna y el tesinista Pablo Márquez, fueron publicados en 2020 en la revista no arbitrada BioRxives, y están actualmente en revisión para su publicación en la revista Genome Research. (Berna L, Márquez P, **Cabrera A**, Greif G, Francia ME*, Robello C*. (2020) Reevaluation of two Apicomplexans genomes reveals misassembly, karyotype differences and lack of synteny. bioRxiv 2020.05.22.111195; doi: https://doi.org/10.1101/2020.05.22.111195. En Revisión en Genome Research).

Reevaluation of the *Toxoplasma gondii* and *Neospora caninum* genomes reveals misassembly, karyotype differences and chromosomal rearrangements

Luisa Berna[1], Pablo Marquez[1], Andrés Cabrera[1], Gonzalo Greif[1], María E. Francia[2,3,*], Carlos Robello[1,4,*]

AUTHORS AFFILIATIONS

[1] Laboratory of Host Pathogen Interactions-Molecular Biology Unit. Institut Pasteur de Montevideo. Montevideo, Uruguay
[2] Laboratory of Apicomplexan Biology. Institut Pasteur de Montevideo. Montevideo, Uruguay.
[3] Departamento de Parasitología y Micología. Facultad de Medicina-Universidad de la República. Montevideo, Uruguay.
[4] Departamento de Bioquímica. Facultad de Medicina-Universidad de la República. Montevideo, Uruguay.
*Corresponding authors

ABSTRACT

Neospora caninum primarily infects cattle causing abortions with an estimated impact of a billion dollars on worldwide economy, annually. However, the study of its biology has been unheeded by the established paradigm that it is virtually identical to its close relative, the widely studied human pathogen, *Toxoplasma gondii*. By revisiting the genome sequence, assembly and annotation using third generation sequencing technologies, here we show that the *N. caninum* genome was originally incorrectly assembled under the presumption of synteny with *T. gondii*. We show that major chromosomal rearrangements have occurred between these species. Importantly, we show that chromosomes originally annotated as ChrVIIb and VIII are indeed fused, reducing the karyotype of both *N. caninum* and *T. gondii* to 13 chromosomes. We reannotate the *N. caninum* genome, revealing over 500 new genes. We sequence and annotate the non-photosynthetic plastid and mitochondrial genomes, and show that while apicoplast genomes are virtually identical, high levels of gene fragmentation and reshuffling exists between species and strains. Our results correct assembly artifacts that are currently widely distributed in the genome database of *N. caninum* and *T. gondii*, but more importantly, highlight the mitochondria as a previously oversighted source of variability and pave the way for a change in the paradigm of synteny, encouraging rethinking the genome as basis of the comparative unique biology of these pathogens.

INTRODUCTION

The Apicomplexa comprise a large phylum of parasitic alveolates of medical and veterinary importance, causing deadly diseases such as malaria, criptosporidiosis, neosporosis and toxoplasmosis, among others. With the exception of a few commonalities such as their obligatory intracellular lifestyle, the presence of specialized secretory organelles and of secondary endosymbionts, the apicomplexans differ greatly in

morphology, host range specificity, pathogenicity, reproductive strategy and transmission. Understanding the molecular basis of these differences has been the focus of much research. Comparative genomic analyses revealed that, albeit all small, apicomplexans genomes vary greatly in size, ranging from 9 to 130 Mb[1,2]. Having diverged from a common ancestor 350-824 million years ago[3], shy of 900 genes are conserved amongst them, whereby major genomic rearrangements can be observed[1].

High synteny, defined as conserved content and order of a given genomic locus, is rarely observed[1]. A seemingly stark exception to this are the genomes of *Toxoplasma gondii* and *Neospora caninum.* Morphologically, these parasites are virtually indistinguishable, so much so, that *N. caninum* was only recognized as a separate species in 1988[4,5]. Moreover, both species exhibit similar tropism within their hosts, where they can infect virtually any nucleated cell. They both exhibit a fast replicating form (tachyzoite) causing acute disease, that transitions into a slow dividing form (bradyzoite) which persists in immune-privileged sites, such as the brain, establishing chronic infection. In line with this, initial comparative analysis concluded that these species have largely conserved genomic content, and are largely syntenic[6]. Despite their commonalities, however, the biology of these pathogens also differs significantly. *T. gondii* infects a wide range of intermediate hosts, including humans, causing deadly disease in immunocompromised individuals or by congenital transmission. In contrast, *N. caninum* infects primarily cattle, causing abortions with an estimated impact of a billion dollars on worldwide economy, annually[7]. Feline species act as definitive hosts of *T. gondii* whilst sexual replication of *N. caninum* occurs only in canids [8–10]. These biological differences have been largely ascribed to absence, point mutations and pseudogenization of *T. gondii* virulence factors in *N. caninum*, and the comparative amplification of surface protein-coding gene families in *N. caninum*[6,11].

Advancements in genome sequencing technologies have accompanied the fast-paced genomics era. Particularly, third generation sequencing technologies, such as Pacific Bioscience (PacBio) and Oxford Nanopore sequencing outperform prior technologies by providing very long reads that can span regions containing repetitive sequences. This has led to improvements in the assembly of previously unattainable genomes, such as those presenting high proportions of repetitive sequences, allowing whole new genomes to be assembled with high accuracy. Here, we set out to sequence and *de novo* assemble two *N. caninum* strain's genomes and the *T. gondii* genome, using PacBio and Oxford Nanopore. Our *de novo* assembly of the three genomes identifies largely incorrectly assembled reference genomes. Strikingly, our work reveals that previously annotated chromosomes VIIb and VIII are in fact a single chromosome in both species, reducing the total number of chromosomes in these coccidia to 13. Greater sequencing coverage and corrected assembly uncovers major chromosomal rearrangements, hundreds of previously unidentified *N. caninum* genes, and more accurate account for gene copy number. In addition, we fully annotate the previously uncharacterized apicoplast genome of *N. caninum*, and note large levels of mitochondrial heteroplasmy exist in both species. More importantly, our results ultimately challenge the paradigm of gene content synteny between *T. gondii* and *N. caninum*, prompting us to further explore these species unique genome structures as potentially overlooked sources of important biological information.

MATERIAL AND METHODS

Cell culture

N.c. Liverpool was acquired from ATCC (50845). *N.c.Uru1* was isolated from a local congenitally infected calf as described in ([12]). *T. gondii* RH ΔKu80[13] strain was kindly provided by Boris Striepen. All strains were maintained and grown as described in Cabrera, et. al. 2019[12].

High Molecular Weight DNA extraction and sequencing

For "Oxford Nanopore" and Illumina sequencing, DNA was extracted from *N. caninum* Liverpool, *N. caninum* Uru1 and *T. gondii* RH ΔKu80[13] strain, using a DNA purification kit from Zymo Research (#D4074). Minion Oxford Nanopore sequencing was done *In house* as described in [14]. Sequencing libraries were prepared using a ligation sequencing kit and a Native barcoding expansion kit (EXP-NBD103/SQK-LSK108, Nanopore, England) according to ([15]), starting from 1 µg of total genomic DNA. 12 hours sequencing was performed in an R9.4 Flow Cell (FLO-MIN106, Oxford Nanopore). Base calling and sequence retrieval was done using Guppy basecaller version 3.0.3. For PacBio sequencing, DNA was extracted from *N. caninum Liverpool*, *N. caninum Uru1* and *T. gondii* RH ΔKu80[13] strain by overnight incubation in lysis buffer (Tris-EDTA buffer supplemented with 1 ug/mL of RNAse A, 10% SDS, 20% Proteinase K) at 55°C, phenol/chloroform extraction, ethanol precipitation and resuspension in ultrapure water. PacBio Sequencing was performed at the Integrative Genomics Core (Beckman Research Institute, Monrovia, CA.), using 5 SMRT cells per sample for *N. caninum* and *T. gondii* genomes. Illumina sequencing of *N. caninum* DNA was also done at the Integrative Genomics Core to 65x coverage.

Genome assembly and annotation

PacBio reads were assembled using HGAP Assembly software[16]. Oxford Nanopore reads were assembled using CANU[17,18]. Assemblies were merged using Quickmerge software[19]. A single conflict region in the assembly was solved by PCR using primers: M1_F: GAGGCGCTTACAATCAACCC, H37_F_M1_R: GAGACAGGACGGACTGAAGA, H37_R: CTGCTCTGTCTGAACAGGTT, M37_F: GCGAACAGCACGAAGTGAGA, M37_R: TCGTGCTTTGAGCATCCTCT. Short nsertions and deletions, a common artifact produced by long read technologies, were corrected using illumina reads in Pilon[20]. Reads used include our own, from DNA purified as described above, as well as raw reads obtained from Sequence Read Archive (SRA) NCBI repositories (Accession IDs: PRJNA531306, ERR012899 and ERR012900). Gene annotation was performed using the automated annotation tool COMPANION[21] using Agustus Threshold 0.2, Taxon ID 5811, Align reference proteins to target sequence as parameters (others as default), and supporting RNA seq data to produce a transcript assembly. This was first aligned with Cufflinks[22] and assembled with TopHat[23] using SRAs with accession IDs ERR690607, ERR690608, SRR4013168, SRR4013169, SRR4013170, SRR4013171, SRR4013172 and SRR4013173 as input sequences. Apicoplast and mitochondrial genomes were identified by manual GC filtering and confirmed by Blast. Apicoplast genome was annotated using Mfannot[24]. Mitochondrial genome annotation was done in MITOS (http://mitos2.bioinf.uni-leipzig.de/index.py) using Opisthokont as reference. PacBio and MinION data has been deposited in NCBI repository (BioProject ID PRJNA597814).

Comparative Genomic Analysis

Assembled genomes were compared using NUCmer[25] to create the alignments between the assemblies being compared and Assemblytics[26] for visualization. Plots comparing the synteny between assemblies were obtained with the visualization tool Circos[27], using the output from Blast to create links between chromosomes. Repetitive regions were analyzed using YASS[28]. Individual chromosome comparisons were performed with Artemis[29] and ACT. IGV[30] was used for visual inspection of aligned reads (wgs and rna-seq) on the assemblies. Specific scripts generated in this study were written in R environment[31] and Bash to parse results and automate pipelines. Telomeres were identified by searching on chromosome ends for the typical TTTAGGG and AAACCCT septameric repeats. Centromeric regions in *Neospora* were determined by BLAST against previously identified centromere sequences in *Toxoplasma gondii* by ChIP-chip[32]

RESULTS

Neospora caninum and *Toxoplasma gondii* long-read assembly genomes

To assemble the *Neospora caninum* genome, we sequenced DNA from the reference strain *N. caninum* Liverpool (NcLiv), and a recent isolate from an experimental farm in Uruguay, named *N. caninum* Uru1 (NcUru1)[12]. Sequencing was done by Pacific Biosciences technology, Oxford Nanopore and Illumina. Reads derived from each sequencing were assembled independently, and then combined into a single assembly per strain (**SFig 1A**). For *NcLiv*, PacBio and Nanopore assemblies matched completely, with the exception of a single conflict region, which was resolved by PCR (**SFig1B-C**). The assembled genomes were corrected with publicly available Illumina reads or those obtained *in house*. For *NcLiv*, the sequencing resulted in over 100x depth, vastly improving the currently available genome coverage (**Table 1**). Genomes of *N. caninum* strains were assembled separately.

Assemblies for both *Neospora* strains were practically indistinguishable, indicating high genome similarity. *NcLiv* genome consisted of 13 large contigs and 31 short (less than 122kb) unplaced fragments. With an N50 of 6.4Mb, 75% of the 61.6 Mb nuclear genome is in 8 of the largest chromosomes **(Table 1).** The 13 largest contigs correspond to complete chromosomes; both 5' and 3' telomeres were mapped for 10, while we found one telomere at one end and sub-telomeric regions at the other end for 3 (**Supp.Table 1**). Putative centromeric sequences were identified *in silico* either by blasting the *T. gondii* centromeres (Chr Ia, II, IV and V) or by identifying large regions devoid of gene-coding sequences flanked by syntenic genes flanking the centromeric regions of *T. gondii (*Chr Ib, IX and XII*)* (**Fig 1B-E and Supp. Table 2**). Our assembly revealed that, in contrast to what has been reported, the *N. caninum* genome is not organized in 14 chromosomes. Rather, previous ChrVIIb and ChrVIII are in fact a single chromosome (**Fig1 B)**.

Table 1. Metrics of the *de novo* genome assembly of *N. caninum* Liverpool

Nuclear Genome (NcLiv)		
Genome properties		
	Chromosomes	13
	Unplaced contigs	31
	Total length	61.5Mb
	GC (%)	54.8
	N50	6.4Mb
	N75	3.6Mb
	L50	4
	L75	8
	# N's per 100 kbp	0
Protein-coding genes		
	Number of gene models	7540

	Pseudogene	258
	Mean exons by gene	5.8
	Percentage coding	59.2%
	Mean CDS length (pb)	4872
	Mean exonic length (pb)	423
	Coding GC (%) 60.1%	
Intergenic regions		
	Mean Length (pb)	3091
RNA genes		
	tRNA	157
	rRNA genes	22
	snRNA	12
Apicoplast Genome (NcLiv)		
	Contigs	2
	Total length	52.3 Kb
	GC (%)	23.7%
Mitochondrial Genome (NcLiv)		
	Contigs	9
	Largest contig	32.4 Kb
	Total contigs length	121 Kb
	GC (%)	36.8%

Given that the previous *N. caninum* genome assembly had been constructed using as the reference the *Toxoplasma gondii* genome, by assuming that these species conserve synteny and gene order, we next examined how our new *N. caninum* genome assembly compared to that of the *T. gondii* reference genome**(Fig 1A***)*. To our surprise, not only did the newly assembled *N. caninum* genome differ from that of *T. gondii* in the total number of chromosomes, but a number of large chromosomal rearrangements could be observed (**Fig1 B)**. Strikingly, only half of the chromosomes' structure, corresponding to *N. caninum*'s chromosomes Ib, II, III, IX, X and XII, are maintained between these species.

Next, we wondered whether the observable differences between the species were an artifact of the *T. gondii* genome assembly based on shorter reads. To this end, we sequenced the genome of a Type I strain (RH) of *T. gondii* both by PacBio and Oxford Nanopore, and *de novo* assembled the genome. Our long read sequencing resulted in a 108x coverage, and its assembly closely matched that reported by using Sanger and 454 sequencing (**Fig1 C)** confirming that the differences observed with *N. caninum* are not

artifactual. However, we were able to once again observe the fusion of chromosomes VIIb and VIII in *Toxoplasma*, thereby indicating that the karyotype for both these apicomplexans is 13, and not 14 as previously reported (**Fig1 D)**.

FIGURE 1

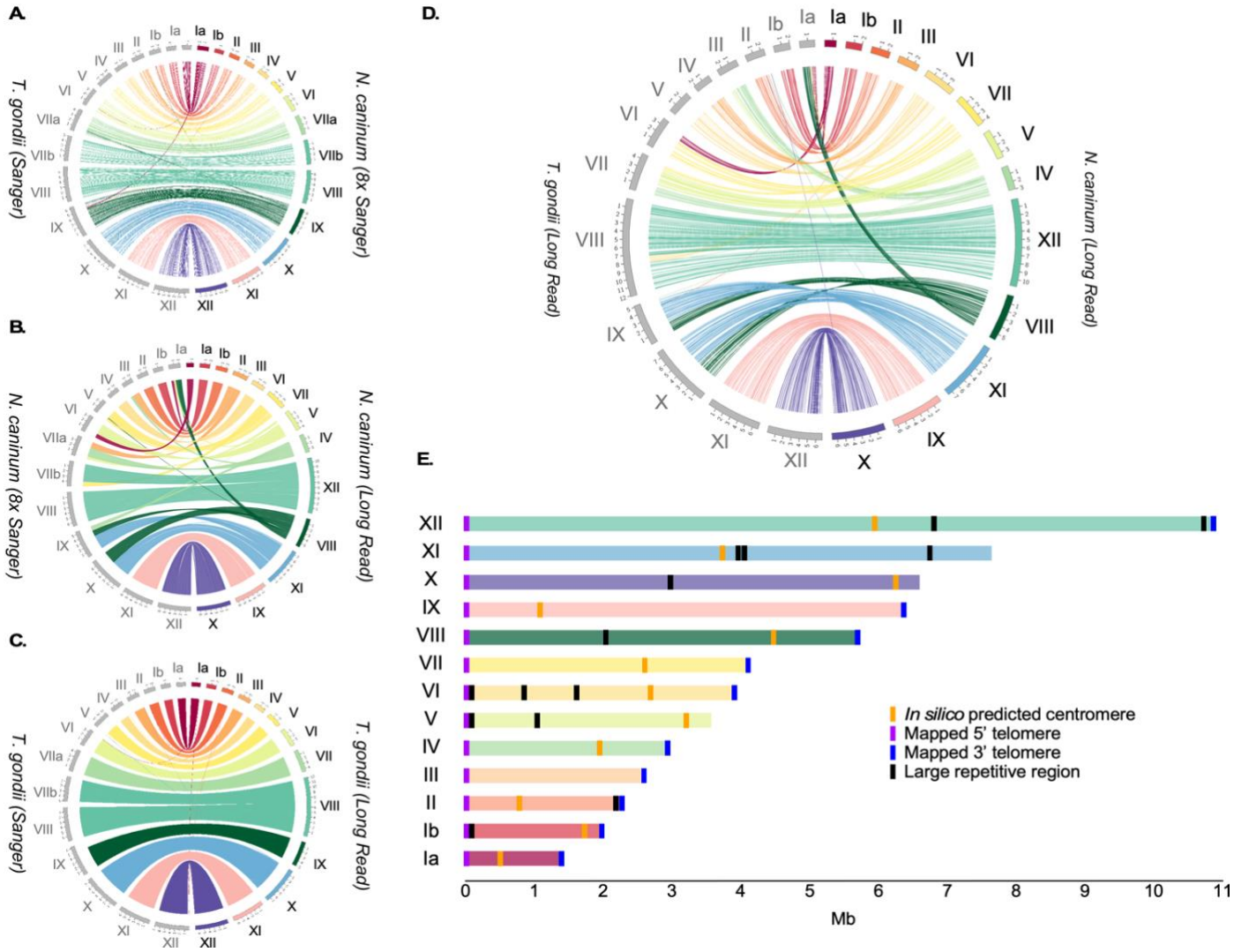


Figure 1. **Comparative analysis of genome assemblies of *Neospora caninum* and *Toxoplasma gondii* using third generation sequencing data reveals misassembly and karyotype differences. A**. Comparative analysis of the *Toxoplasma gondii* type II (TgME49) genome assembly and the *Neospora caninum Liverpool* strain genome assembly, obtained based on Sanger technology sequencing data. **B**. Comparative alignment of the *Neospora caninum Liverpool* genome assemblies using Sanger and third generation (long read) technology. **C**. Comparative alignment of the *Toxoplasma gondii* type II (TgME49) genome assemblies based on Sanger technology sequencing data or third generation (long read) technology of *Toxoplasma gondii* type I (TgRH) . **D.** Comparative alignment of the *Toxoplasma gondii* type I (TgRH) and the *Neospora caninum Liverpool* genome assemblies based on third generation (long read) sequencing technology. **E.** Chromosomal layout of Neospora caninum. Karyotype, chromosome length, telomeres, putative centromeres and large repeats, are shown.

We next explored whether the breaks in synteny between these closely related species correlated with any distinguishable genomic features. We noticed that the level of

sequence identity between the species varies along chromosomes. Coding regions seem to be highly conserved (82.4%). Nonetheless, we noticed that there is a detectable shift in codon usage between the two species, whereby *N. caninum* tends to use GC richer codons than *T. gondii.* The difference in codon usage conveys a detectable difference in composition whereby the mean GC content for N. caninum is 58.3% while it is 56.4% for *T. gondii*, being this a statistically significant difference (*p-value* $1.4x10^{-9}$). The same trend can be observed for GC3 (63.7% and 61.0%, respectively) and less markedly for GC1 (**SFig 2**). On the other hand, non-coding regions, including intronic and intergenic regions are on average 80.7% identical. Non-coding regions throughout the genome of *N. caninum* are interspaced with low complexity repetitive sequences rich in A/G or A/T. In addition, we identified three conserved sequence motifs present at a number of regions where synteny is interrupted. Particularly, five chromosomes (IV-VIII) exhibit comparative chromosomal rearrangements at regions that coincide with repetitive units of such domains (**Fig2A and B,** and **Supp. Table 3**).

FIGURE 2

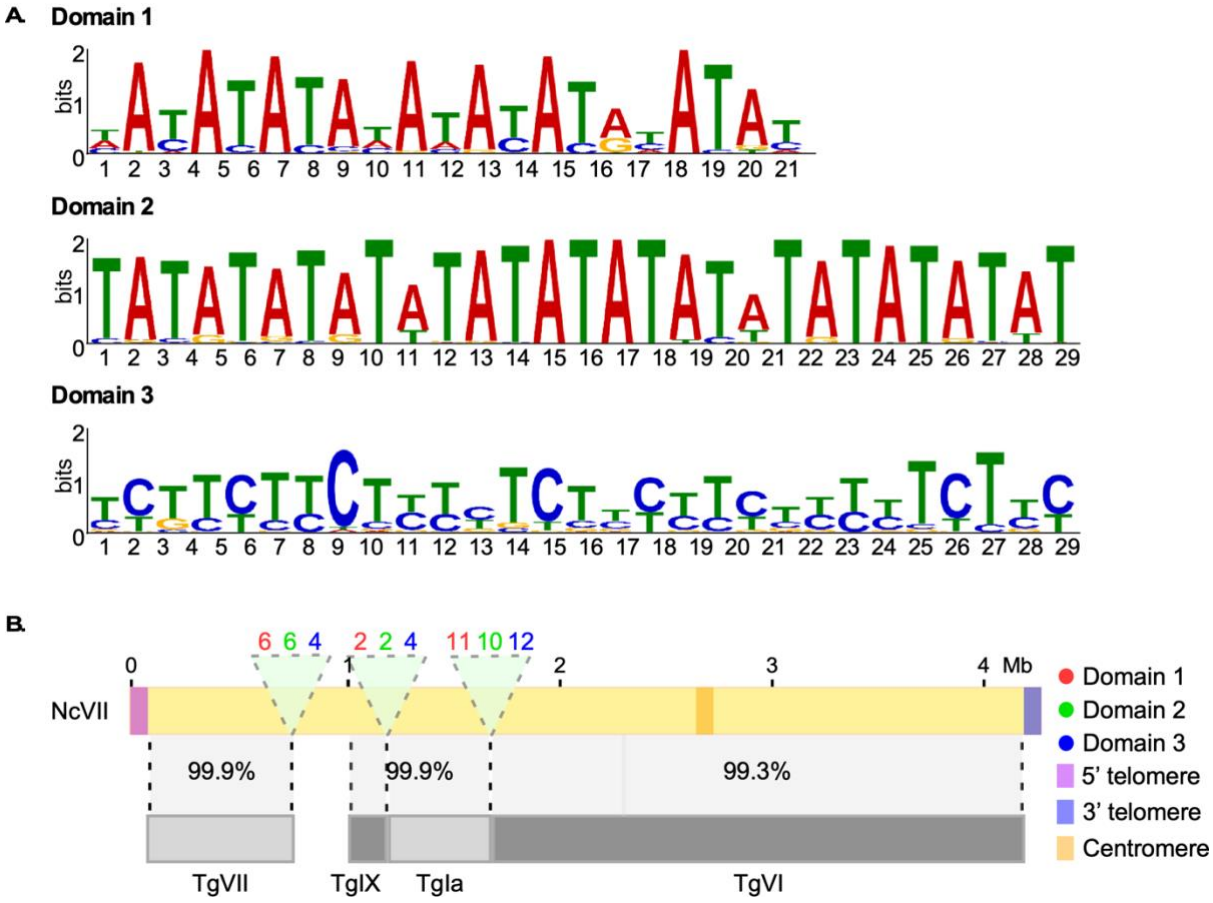


Figure 2. **Regions of synteny breaks between *N. caninum* and *T. gondii* are populated by three conserved domains**. **A.** Sequence identity of domains identified at regions where chromosomal rearrangements have occurred. **B. Graphical representation of chromosome VII of *N. caninum Liverpool***. Comparative alignment to the *Toxoplasma gondii*

chromosomes. Percentages of sequence identity are shown. Regions examined for the presence of motifs are indicated (a, b, and c, light green). The position of the putative centromere is indicated in orange. Note that large repetitive regions were not identified in this chromosome. 5' (light purple) and 3' (dark purple) telomeres are indicated. Identity and number of domains found per region, in chromosome VII, are indicated.

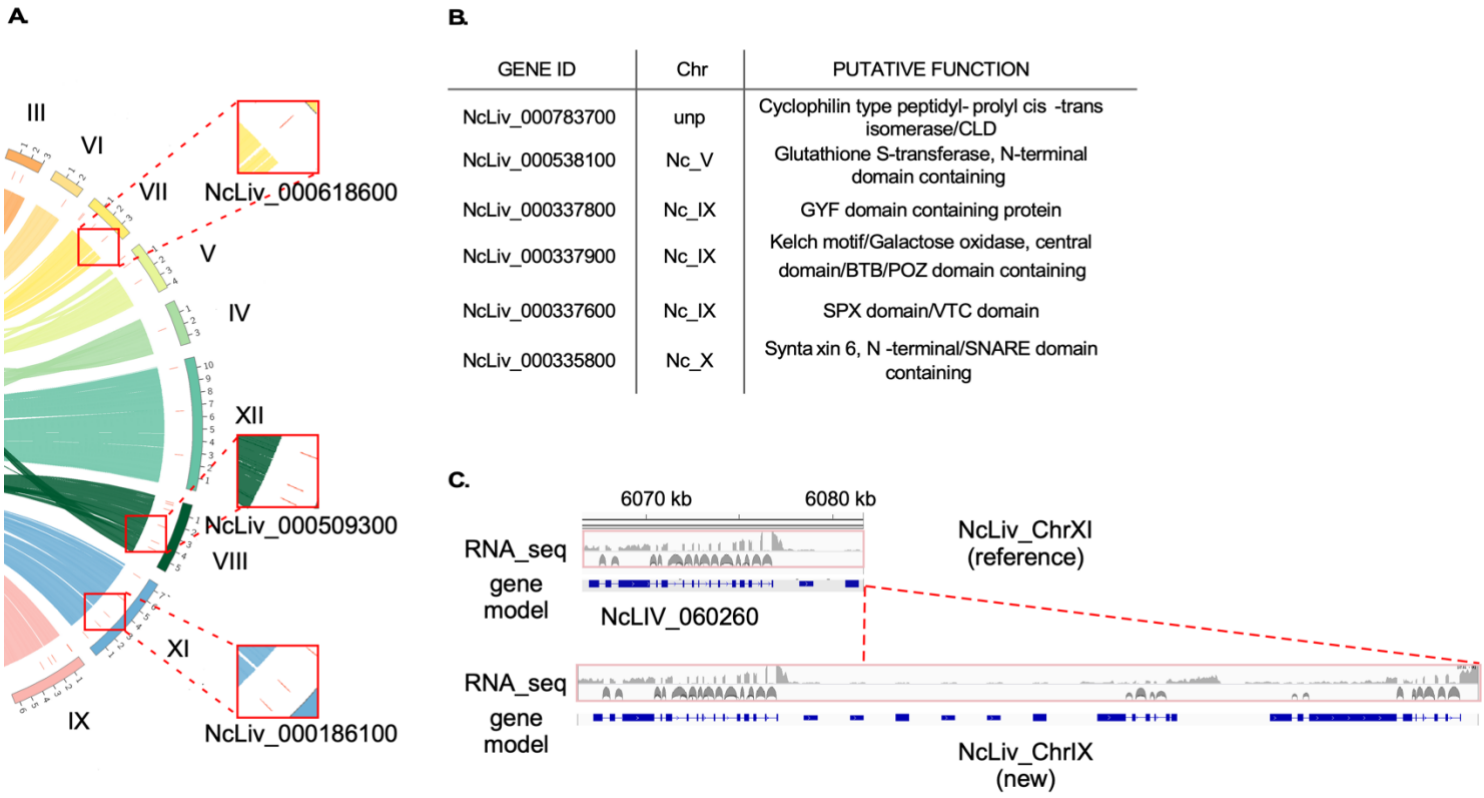
Gene Annotation

Annotation of the newly assembled NcLiv genome, using homology-based searches and RNA-seq datasets, resulted in the annotation of 7540 genes and 254 pseudogenes. Of these, 7502 are distributed in 13 chromosomes while 38 are in unplaced sequences. The products of 4553 genes have a putative assigned function, while 2987 code for hypothetical proteins. Comparatively, 554 new genes were identified in our annotation; 9 are coded for in unassembled sequences, while 545 are distributed in the chromosomes. Albeit not originally annotated, 516 of these can be found in the original Sanger sequence reads of the *NcLiv* genome, with over 95% identity in over 95% of the gene's length. Of these, 494 mapped to the originally assembled chromosomes, while 22 belong to unplaced sequences.

No common theme was observed among the newly annotated genes. Of these, the most frequently found are SRS domain-containing proteins, followed by WD-domain, and zinc-finger containing proteins. This is consistent with previous findings showing that the SAG-related family of surface proteins is amplified in *N. caninum*[6]. Previously, 227 members of this family had been identified. Here, we annotate a total of 231 SAG-related proteins. Finally, 38 novel genes were uniquely identified in the newly sequenced genome; 36 are distributed in 11 of the assembled chromosomes, while 2 remain in unplaced sequences (**Fig3 A**). Of these, 6 have putative assigned functions or recognizable domains; a Syntaxin 6, N-terminal/SNARE domain containing protein, an SPX domain/VTC domain containing protein, a Kelch motif/Galactose oxidase, central domain/BTB/POZ domain containing protein, a glutathione S-transferase, N-terminal domain containing protein, and a Cyclophilin type peptidyl-prolyl cis-trans isomerase (**Fig3B and Supp. Table 4**). The products of the remaining 32 genes are regarded as hypothetical. We note that many of this new genes resulted either from the annotation of sequencing gaps (**Fig 3A**) or sequencing that extended through repetitive regions where previous sequencing had failed (See **Fig3C** for a representative example)

Next, we explored whether the new annotation revealed presence of uncharacterized virulence factors. We surveyed the genome for homologs of major virulence factors characterized in *T. gondii*, the majority being kinases involved in protecting the parasitophorous vacuole from host-cell intrinsic defenses; ROP5, ROP16, ROP17, ROP18 and ROP38, dense granule secreted effectors such as GRA16, GRA24, GRA25 and GRA44, as well as, IST. No major differences were found between the annotations regarding virulence factors with the exception of ROP38, whereby 4 gene copies had been reported, we could resolve 9 ROP38 copies arranged in tandem. Our annotation is consistent with previous reports describing the absence of GRA24 and IST homologs in *N. caninum*.

FIGURE 3



GENE ID	Chr	PUTATIVE FUNCTION
NcLiv_000783700	unp	Cyclophilin type peptidyl- prolyl cis -trans isomerase/CLD
NcLiv_000538100	Nc_V	Glutathione S-transferase, N-terminal domain containing
NcLiv_000337800	Nc_IX	GYF domain containing protein
NcLiv_000337900	Nc_IX	Kelch motif/Galactose oxidase, central domain/BTB/POZ domain containing
NcLiv_000337600	Nc_IX	SPX domain/VTC domain
NcLiv_000335800	Nc_X	Synta xin 6, N -terminal/SNARE domain containing

Figure 3. **Gene annotation reveals previously unknown genes in the genome of *N. caninum*. A.** Graphical representation of the position of novel genes in the newly assembled chromosomes. Red lines mark the position of novel genes along chromosomes. Alignment to previously assembled NcLiv genome is partially shown for reference. Three insets are shown to highlight the annotation of three new genes in three newly assembled genomic regions. **B.** Putative function of xix out of the 38 newly identified genes. The remaining 32 genes are annotated as hypothetical**. C.** Representative example of the improvement in annotation in regions that had been previously collapsed due to the presence of tandem repeats. Several new genes were annotated, all of whose annotation is supported by RNA-seq data.

Neospora caninum apicoplast genome

The apicoplast is a relic non-photosynthetic chloroplast-like organelle of bacterial origin present in most apicomplexan parasites, whereby essential lipid synthesis occurs[33,34]. This organelle is of great importance as it has been validated as the target of anti-parasitic drugs such as clindamycin[34]. Most apicoplast proteins are encoded for in the nucleus, and later imported[35–41]. However, the apicoplast harbors its own genome, which has been traditionally regarded as coding for proteins needed for its maintenance[42].

Surprisingly, we identified two contigs of 44 and 26kb, with markedly lower GC content than that of the nuclear genome (21.5 vs. 59.2%), and a 10kb inverted repeat sequence (**Fig 4A-H**). Annotation of these revealed 60 open reading frames most of which correspond RNA polymerase subunits and ribosomal proteins. In addition, the coding

regions for SulfB and Clp protease, and a hypothetical protein were identified (**Fig4C, E** and **Supp. Table 5**). These have been shown to be resident proteins of the apicoplast, thereby indicating these two contigs correspond to the apicoplast genome of *N. caninum*.

The apicoplast genome has been reported to be circular. However, we do not find compelling evidence to favor circularization of the apicoplast genome over a two linear chromosome model, in spite of using long reads, regarded as optimal to resolve these kinds of conundrums (**Fig 4I and SFig 3**). The assembled sequences bear significant homology at their ends, allowing their collapse into a single molecule of 56kb. However, mapped reads to this pseudomolecule do not support its circularization **(Fig4I and SFig 3C)**. This could be explained by lower sequencing coverage of the apicoplast genome due to its markedly lower GC content, or alternatively, it could suggest that the genome is indeed linear.

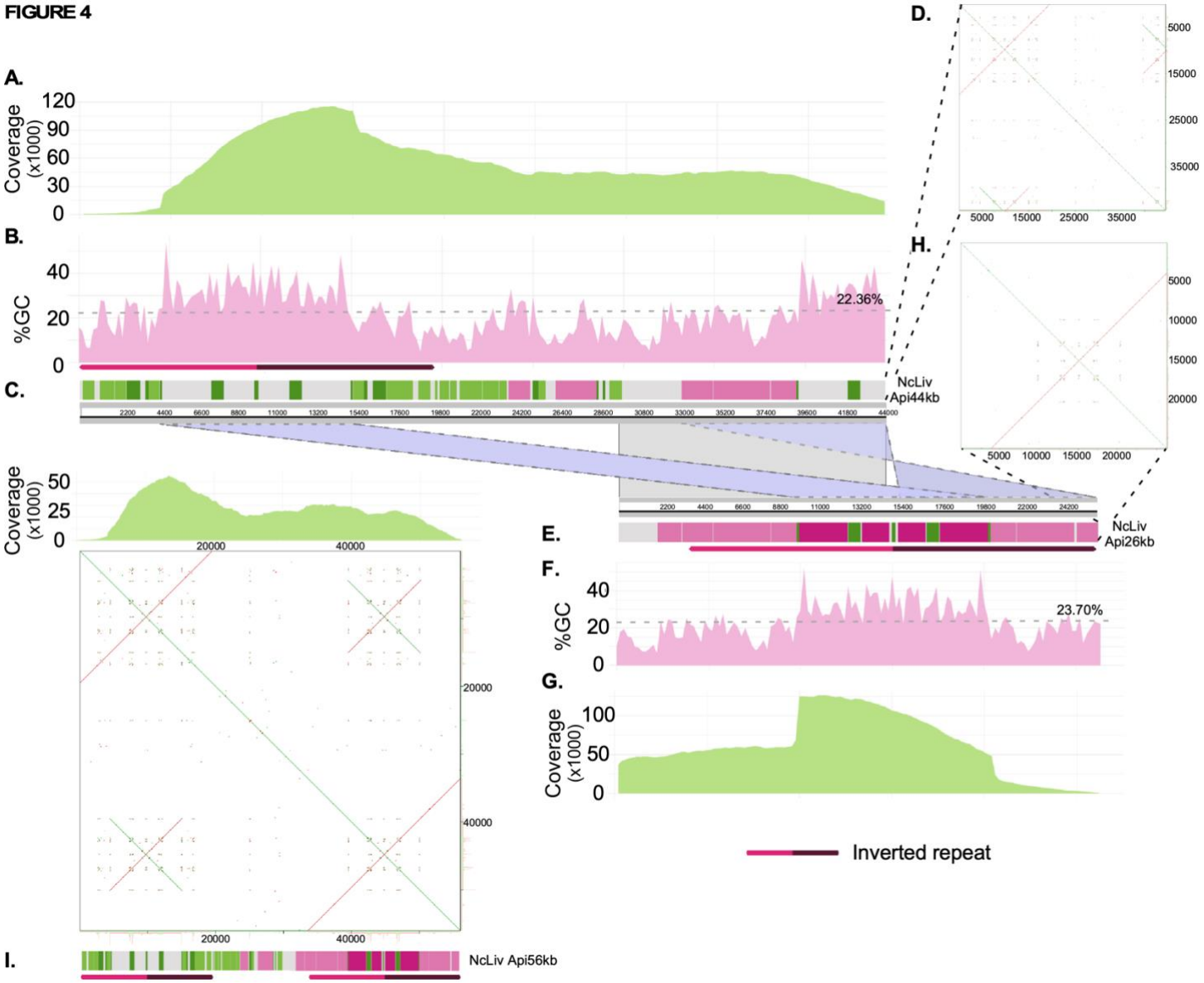


Figure 4. ***Neospora caninum*** **Apicoplast genome structure and annotation. A.** Read Coverage count along the length of the 44kb apicoplast genome contig. **B.** Percentage of GC

along the length of the 44kb apicoplast genome contig. Average GC is shown above the dotted line. **C.** Annotation along the length of the 44kb apicoplast genome contig (see supplementary Table 5 for details) **D.** The presence of an inverted repeat sequence at the 5' end of the contig is graphically represented in a YASS plot. **E.** Annotation along the length of the 26kb apicoplast genome contig (see supplementary Table 5 for details) **F.** Percentage of GC along the length of the 26kb apicoplast genome contig. Average GC is shown above the dotted line. **G.** Read Coverage count along the length of the 26kb apicoplast genome contig **H.** The presence of an inverted repeat sequence at the 3' end of the contig is graphically represented in a YASS plot. **I.** Graphical representation of the apicoplast genome structure if both contigs are fused together Read Coverage count supporting the fusion, along the length of the resulting 56kb apicoplast genome contig, are shown. Note that both annotation and the position of the inverted repeat sequences are shown. The YASS plot graphically shows the presence of both the 5' and 3' inverted repeats. Note, however, that the presence of this highly similar repetitive elements at both ends is not sufficient to support circularization of the genome.

Mitochondrial genomes

A number of large contigs (up to 32.4kb) amounting to a total of 121kb were identified upon sequencing *NcLiv* (**Table 1, Fig5 and SFig 4**). These contain sequences with homology *to* classically known mitochondrial genes such as cox1 and cob1, and ribosomal RNAs (**SFig 4 and Supp Table 6**). In addition, these contigs display a GC content averaging 36.8%, markedly lower than that of the nuclear and apicoplast genomes (**Table 1**). Interestingly, we could not assemble these contigs together, and neither one of them circularizes on its own. All of these contigs contain fragments of cox1 and cob1 open reading frames, however, the vast majority of them are interrupted by internal STOP codons. Three of the contigs feature replication origins of the heavy strand (OH) sequences, and only one of them features a potentially functional cox1 gene with no internal STOPs. One other contig lacks an origin of replication but features a potentially functional cob1 copy (**Supp Table 6**).

The mitochondrial genome of *NcUru1* is distributed in 20 contigs, which vary in size ranging from 1437bp to 86kb with a medium of 12kb in size, and average a GC contents of 36.95%. The largest contig, of 86 kb, features three origin of replication (OH sequences), 36 fragments with homology to cytochrome b, 56 fragments with homology to cytochrome c oxidase subunits I and III, and several ribosomal RNA encoding genes. All genes encoding for cob, cox1 and cox3 contain internal STOP sequences which would result in truncated proteins. Likewise, both genomes encode for the intronic endonuclease LAGLI.

Interestingly, while apicoplast genomes are 95% identical between the two *N. caninum* strains sequenced, the contigs corresponding to the mitochondrial genomes of *NcUru1* and *NcLiv* did not coincide with each other (**Fig5**). They both showcase a similar structure consisting of multiple linear contigs, featuring pseudogenized copies of cox1 and cob1, whose order seems to reshuffle in every contig (**Fig5**). The gene order, distribution and length is unique to each strain.

Twenty nine contigs ranging from 1.1 to 39 kb in size, with a median of 8.0kb were identified as the mitochondrial genome of *T. gondii*. The contigs' GC content averages 36.6%. A similar structure of shuffled pseudogenes can be observed, whereby multiple copies of different size coding fragments of cob, cox1 and cox3 populate the mitochondrial

chromosomes, together with rRNAs *(***Fig5**). Origins of replication of the heavy strand (OH sequences) can be identified in six contigs. Multiple coding sequences for the intronic endonucleases GIY and LAGLI can be identified in six contigs. Strikingly, both between *N. caninum* strains, or between *N. caninum* and *T. gondii*, no single contig bearing the same combination or order of gene coding fragments could be identified (**Fig 5**).

FIGURE 5

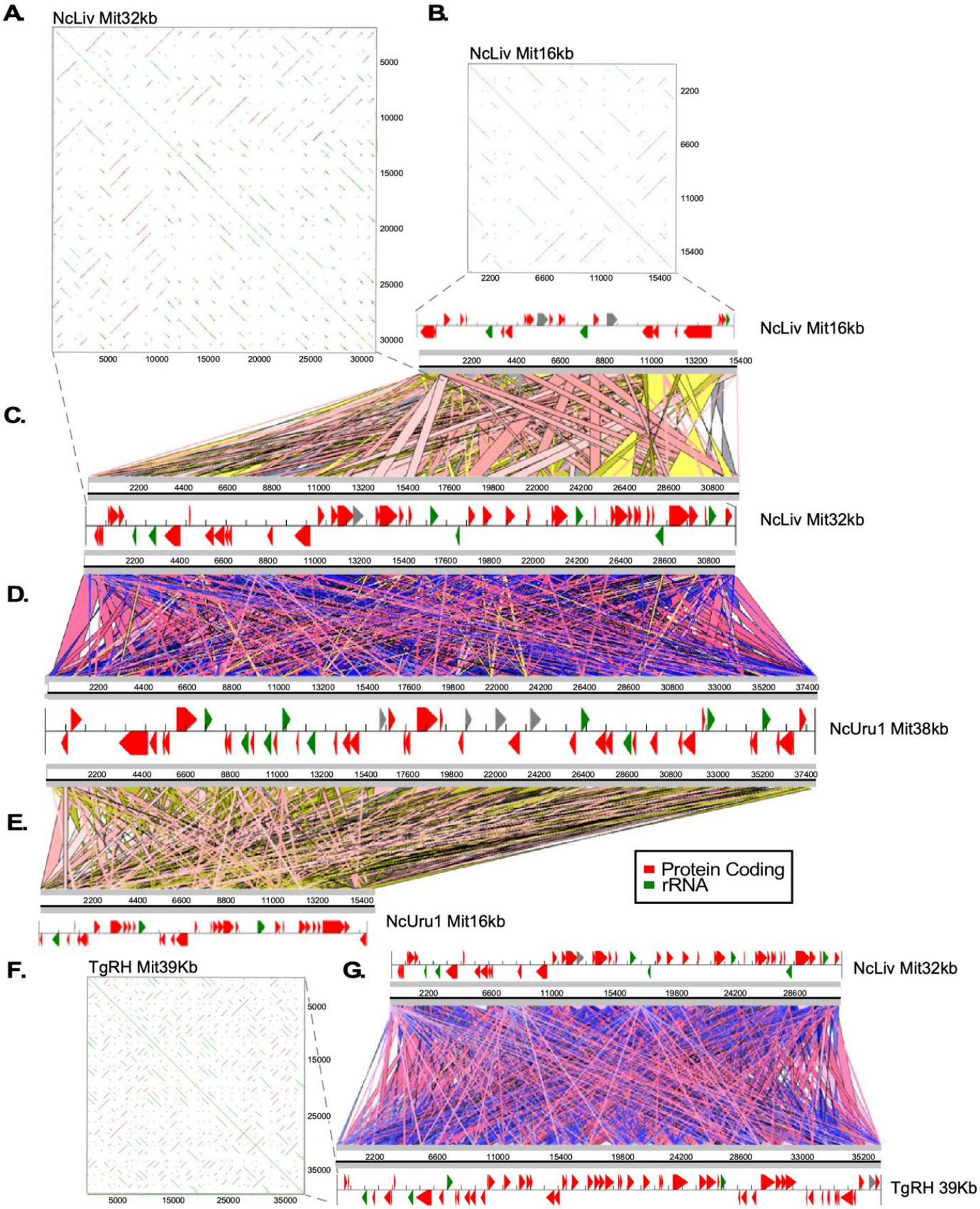


Figure 5. **Comparative analysis of mitochondrial genome structures and annotations of *Neospora* and *Toxoplasma* reveals gene fragmentation and reshuffling between species and strains. A.** The repetitive nature of the gene structure in a 32kb mitochondrial DNA contig of *Nc Liverpool* is graphically represented in a YASS plot. **B.** The repetitive nature of the gene structure in a 16kb mitochondrial DNA contig of *Nc Liverpool* is graphically represented in a YASS plot. **C.** Comparative alignment between two NcLiv mitochondrial contigs of 16 and 32kb, respectively. **D**. Comparative alignment between an NcLiv mitochondrial contigs of 32kb and an NcUru1 mitochondrial contig of 38kb. **E**. Comparative alignment between two NcUru1 mitochondrial contigs of 16 and 38kb, respectively. **F.** The repetitive nature of the gene structure in a 16kb mitochondrial DNA contig of *NcUru1* is graphically represented in a YASS plot. **G.** Comparative alignment between an *NcLiv* mitochondrial contigs of 32kb and an *T. gondii* mitochondrial contigs of 39kb.

DISCUSSION

Our ability to sequence genomes and annotate genes have greatly enhanced our understanding of the molecular basis of life, health and disease. Comparative genomics has allowed us to establish evolutionary relationships among living organisms, and aided in the development of specific molecular diagnosis and the rational prediction of selective drug targets. Widely used sequencing technologies, such as Sanger, 454 and Illumina, have played a pivotal part in these advancements. However, the limitations of these technologies, namely their trouble reading through repetitive regions and their short read outputs, have led to assembly artifacts that are currently widely distributed in genome and proteome databases[43]. A number of protozoan parasite genomes have been recently revisited using third generation sequencing technologies. One noteworthy example is the genome of *Trypanosoma cruzi*, the causative agent of Chagas disease. This genome was massively improved for several strains. Its assembly went from being highly fragmented, in over 4000 short contigs, to less than 2000 contigs, doubling the overall genome size[44,45]. Such improvements have allowed accurate account for gene copy number and the identification of an underlying genome structure. Such advances are essential in order to profiting from the advent of highly specific “guided” genome editing technologies, such as CRISPR/Cas9 technology, understand virulence linked to gene copy number, and inter and intra-species differences.

Here, we disentangled the genomes of two closely related species; *Neospora caninum* and *Toxoplasma gondii* by *de novo* assembling them. The *N. caninum* genome had been previously assembled under the presumption that they were largely syntenic, using short reads[6]. We uncovered that even though half of the genome is indeed structured quite similarly between *N. caninum* and *T. gondii,* seven out of thirteen chromosomes differ significantly from each other. Importantly, our unbiased assembly of both genomes revealed that, strikingly, the karyotype of these apicomplexans is of 13 chromosomes. Our results suggest that chromosomes previously mapped as ChrVIIb and VIII are, in fact, a single chromosome. A number of previous findings support this fusion. Several linkage analysis assays to uncover virulence factors demonstrated that these chromosomes always segregate together[46,47]. Genome interactions mapped by Hi-c analysis revealed chrVIIb and chrVIII had a higher number of interactions with each other than any other combination of chromosomes did. The latter study also showed that the number of contacts between the right telomere of chrVIIb and the left telomere of chrVIII were the highest of all, suggesting

that these could be physically linked[48]. In addition, identification of the centromere of ChrVIIb was unattainable by ChIP-Chip or ChIP-seq[32,49].

Interestingly, we detect a large inversion in ChrXII of *N. caninum* with respect to the *T. gondii* chromosome. This inversion is suggested in the 3D analysis of genome structure done by Hi-C using the *Toxoplasma gondii* Type II strain ME49[48]. However, we do not detect such inversion in our *T. gondii* RH genome assembly suggesting that the ChrXII inversion could be strain-specific.

Genome structure differences and rearrangements are widely observed amongst apicomplexans[1]. However, the driving forces of these differences are ill understood. It is well established that genomic divergence amongst Trypanosomatids, for example, can be partially ascribed to the presence of transposable elements within their genomes. Apicomplexans' genomes, however, are devoid of such sequences.[1] We identified low complexity, repetitive regions but their appearance did not correlate with recombination prone chromosomes, but were rather evenly distributed throughout the genome. We did, however, identify a number of repetitive motifs frequently located in the vicinity of regions where synteny is lost. Experimental validation of these motifs as drivers or "soft spots" for recombination would be needed to mechanistically link them to chromosomal rearrangements.

Unlike the situation for *T. gondii* whereby multiple strains have been fully sequenced, whole genomes of *N. caninum* were so far limited to the reference strain *N. caninum Liverpool*. Detailed population genomics studies based on whole-genome sequences from multiple strains worldwide are lacking, and so is our current understanding of population genetic structure of *Neospora.* Very recently, however, a study analyzing 19 linked and unlinked genetic markers of 50 isolates collected worldwide resolved a single genotype of *N. caninum*[50]. This is consistent with our results whereby our whole genome assembly of NcUru1 is practically indistinguishable from that of NcLiv, despite deriving from completely different geographical locations (Europe vs. South America). Nonetheless, it is well established that great genetic variability exists amongst *N. caninum* strains in the form of SNPs at particular loci and that these genetic differences underlie phenotypic variability[12,51,52]. In this context, it is noteworthy that linkage assays, whereby these minimal differences can be correlated to virulence phenotypes, rely on correct genome assemblies.

The apicoplast is a validated drug target to fight apicomplexan caused diseases such as toxoplasmosis and malaria[34,53]. Despite its importance, however, very few complete apicomplexan apicoplast genome sequences have been reported. The *N. caninum* plastid genome sequence had not been identified prior to this study. However, plastid genome physical characterization suggested a size of approximately 35 kb; whereby formation of oligomeric molecules, migrating as linear molecules in approximate multiples of the unit length, was detected[54]. Despite the ample coverage, and average read lengths that comfortably exceeding the putative apicoplast genome size, we were not able to find evidence that preferentially supported a circularize plastid genome of 35kb over two linear molecules adding up to over a 60kb, fostering an inverted repeat on their ends. It is feasible, however, that the comparatively lower GC content of the apicoplast genome hinders its effective sequencing yielding lower coverage. Nonetheless, our sequencing data cannot distinguish between the presence of a circular molecule harboring an inverted repeat or of two linear molecules, of high identity to each other. Further experiments would be required to elucidate this conundrum.

On the other hand, the apicomplexan mitochondrial genome had been described before as consisting of repeated elements of 6-7kb in length[55]. Mitochondrial genomes commonly encode a number of proteins required for its maintenance; part of the translation apparatus, tRNAs, large and small ribosomal RNAs *rns* and *rnl*; membrane associated proteins which catalyze oxidative phosphorylation: cytochrome b (*cob*), subunits of cytochrome c oxidase (*cox*), ATP synthase subunits 6, 8 and 9, subunits of the NADH dehydrogenase complex; and additional ORFs of unknown functions.
Here, we found that the mitochondrial genomes of *N. caninum* code for fragments of cob, cox1, cox3, rrns, rrnl, Lagli and Giy.

Interestingly, while the nuclear and apicoplast genomes are virtually identical between the two *N. caninum* strains sequenced, mitochondria genomes are strikingly different. Noteworthy, different combinations of ORFs are observed between species (*N. caninum* and *T. gondii*), and between strains of *N. caninum*. Mitochondria also differed greatly in size. Varying size mtDNA fragments had been inadvertently observed before for *Eimeria tenella*, whereby southern blot of Cox3 yielded a smear pattern of 4 to 20 kb[56]. In addition, despite our ample sequencing coverage, we did not find any circular molecule, suggesting that the mtDNA is composed of linear fragments. This has been reported for *Babesia*, *Theileria* and *Plasmodium* mtDNA[57,58]. Gene structure organization differences are also observable amongst these closely related hematozoa apicomplexans.

Strikingly, no mitochondrial contig in *N. caninum* nor *T. gondii* contained a fully functional copy of *cox1* or *cob*. Likewise, *Plasmodium* mitochondrial ribosomal RNAs display high degree of fragmentation. It is unclear how these small RNA fragments come together to form a ribosome[59]. A similar gene structure of fragmented gene pieces (divided in modules) has been described in the free living kinetoplastids. The mitochondrial gene modules of the unicellular flagellate *Diplonemid* are separately transcribed, followed by the joining of partial transcripts to contiguous RNAs[60]. Mitochondrial gene editing, whereby transcripts are altered post-transcriptionally to render a functional product, has so far not described for apicomplexans. However, this process is quite common and mechanistically diverse in this organelle in a wide range of species ranging from protozoa to plants. On the other hand, functional copies of mitochondrial proteins could be encoded for in the nuclear genome and imported into the organelle. Protein import is largely used by the apicoplast, whereby proteins required for its metabolic functions are all imported from the nucleus[38–41,61]. Only those proteins required for the maintenance of its genome are fully transcribed and translated within the organelle[42]. Mitochondrial tRNAs, and ATP synthase subunits are known to be imported in *T. gondii*[55,62,63]. Noteworthy, a functional *cox1* copy has been annotated in the *T. gondii* nuclear genome (TgME49_209260), and a homolog is present in the reference NcLiv genome (NCLIV_003650). In addition, nuclear-encoded divergent cox-related proteins have been identified in the mitochondrial proteome of *T. gondii*[64]. Nonetheless, our results pose questions regarding the mechanisms of mitochondrial protein synthesis and transport which merit consideration.

The molecular mechanisms underpinning such high variability of genetic content among mitochondrial genomes are unknown. However, we identified the presence of LAGLI and GIY among the mitochondria encoded genes. Both these proteins are endonucleases encoded for in invasive introns shown to be mobile elements. Intraspecific variability of fungal mitochondrial genomes has been mechanistically linked to the movement of these endonucleases[65]. Likewise, a GIY type endonuclease present in the second intron of the mitochondrial *cytochrome b* gene in the fungus *Podospora curvicolla* was shown to

autonomously transfer from an ORF-containing intron to an ORF-less allele[66]. Our results pave the way for exploring the exact contribution of these endonucleases to genome variability. It remains to be determined, as well, whether the mitochondrial genome variability observed corresponds to a clonal collection of multiple fragment, homogenously present within a population, or whether the contigs assembled represent a cohort of heteroplasmic mtDNA differentially distributed at the population level.

Overall, our results highlight distinct nuclear genomic structures, and a variable mitochondrial genome, as previously unexplored sources of genetic variability among apicomplexans. Variability is not only observed amongst closely related species, but also between strains. Efforts to explore the mechanistic contributions of this variability could shed light onto the molecular underpinnings of virulence related traits ranging from fitness to differences in drug susceptibility.

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AUTHOR DECLARATION

All authors have read and approved the current version of this manuscript and have agreed to its submission

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6.0 Discusión

Para contextualizar el impacto de los diferentes resultados obtenidos en esta tesis de doctorado, comentaremos en primer lugar qué datos se conocían previamente en el Uruguay sobre este patógeno y cuáles fueron los aportes concretos de mi tesis doctoral.

La primera comunicación acerca de la circulación de *Neospora caninum* en Uruguay data de 1997, y es evidenciada mediante la detección de anticuerpos por inmunofluorescencia indirecta (IFI) en un total de 414 perros, encontrándose una seroprevalencia de 20% (Barber et al., 1997). Posteriormente se describió la presencia del parásito en fetos abortados bovinos (Bañales et al., 1999), y los primeros estudios de seroprevalencia de *N. caninun* en el ganado bovino fueron publicados en el año 2004. En este trabajo se evaluaron por IFI 217 sueros bovinos detectando una seroprevalencia total de 60%, evidenciándose también se evidenció una correlación significativa entre la seropositividad para el parásito y el aborto en esa población bovina, mencionando además la detección del agente en dos fetos abortados por inmunohistoquimica (Kashiwazaki et al., 2004). En estudios realizados dos años después en ganado de carne identificaron seroprevalencia en el entorno del 14%. (Bañales et al., 2006). Además se pudo establecer que en ganado lechero la seroprevalencia era del 22% y que la totalidad de los establecimientos tenían al menos un animal infectado en el rodeo (Piaggio, 2006). En este último trabajo se evidenció la seroprevalencia en los perros, que fue de un 41%, pero no se encontró correlación entre esta y la seropositividad en el ganado (Piaggio, 2006). En ese mismo año se detecta que un tercio de los abortos recibidos por el laboratorio de referencia (DILAVE) son causados por *N. caninum* (Easton, 2006). En otro estudio realizado en la región centro del país (Tacuarembó y Durazno) en 39 pequeños establecimientos

lecheros se detectaron sobre un total de 711 sueros, una seroprevalencia del 28% (Furtado et al., 2011).

Los altos valores de prevalencia en el ganado bovino y la alta detección del agente en los fetos remitidos, dejaron en evidencia hace más de una década que las infecciones por *N. caninum* son un problema que afecta significativamente la producción agropecuaria nacional. Sin embargo, cuando comencé esta tesis de doctorado (2016) la problemática de la neosporosis en el país estaba desactualizada, y por otra parte, si bien los métodos de diagnóstico utilizados en Uruguay eran los adecuados, a nivel mundial se introducían los métodos moleculares para la detección del agente y realización de estudios epidemiológicos moleculares.

Es así que el primer aporte de esta tesis de doctorado es la introducción y la aplicación de las nuevas metodologías para la detección molecular del parásito en muestras biológicas en el país. Si bien el protocolo utilizado es una adaptación de una técnica ya utilizada a nivel mundial, es la primera vez que se publica la detección molecular de este parásito en cerebros bovinos y fetos abortados en nuestro país. Aplicación práctica que no sustituye la anatomía patológica e inmunohistoquímica para detectar al parásito pero sí aporta información epidemiológica y valida el diagnóstico sobre la muestra sospechosa.

En este sentido se aumenta con la introducción de la biología molecular la capacidad diagnóstica del parásito a nivel país, fomentando la transferencia de conocimiento a centros de diagnósticos como el INIA y la Facultad de Veterinaria. Es así, que conjuntamente con el equipo de salud animal de INIA-La Estanzuela (INIA-LE), publicamos un artículo donde se implementa la detección molecular, concluyendo que entre el 2015 y 2018, el 29% de los fetos bovinos abortados por incidencia de una enfermedad infecciosa, remitidos a INIA-LE para diagnóstico, fueron por *N. caninum* (Macías-Rioseco et al., 2020). Esos fetos fueron diagnosticados por

inmunohistoquímica, si bien el diagnóstico preciso se basó en la aplicación de las técnicas moleculares mencionadas anteriormente. Esta técnica también se utilizó para comprobar la co-infección de un feto abortado por el virus de la diarrea viral bovina *y N. caninum* (da Silva Silveira et al., 2020b).

Si bien la detección del parásito es de por sí relevante, la introducción de métodos de análisis de la caracterización genética a través de los marcadores moleculares (microsatélites) aplicados en esta tesis de doctorado, tanto en cepas aisladas como en muestras de fetos abortados, nos permitió profundizar en la epidemiología molecular del parásito (Cabrera et al., 2019). Con la puesta punto de esta potente herramienta podemos ahora establecer la genética de las cepas implicadas en diferentes brotes de la enfermedad.

La publicación que surgió de esta tesis doctoral caracterizando genéticamente los aislados, dejo en manifiesto la complejidad y variabilidad genética de las poblaciones de *N caninum*. Describiendo la gran variabilidad entre las cepas regionales y mundiales, agrupándolas en seis grandes grupos a nivel mundial, al igual que un reciente trabajo realizado con otro conjunto de marcadores moleculares (Khan et al., 2019). Esta variabilidad genética que encontramos en *N. caninum* repercutirá en una variabilidad antigénica, si es así, es capaz de afectar la sensibilidad y especificidad de los métodos diagnósticos en la diferentes regiones. En este sentido es necesario incluir las cepas locales en los diferentes métodos de diagnóstico.

A pesar de su importancia, el diagnóstico etiológico del aborto en bovinos representa un desafío técnico para los veterinarios clínicos y laboratoristas, en parte debido a la dificultad de recuperar los fetos abortados en condiciones extensivas de campo. Dicha dificultad limita la posibilidad de detectar tempranamente los vientres abortados y de efectuar la investigación patológica y microbiológica de los abortos (da Silva Silveira et al., 2020a).

Es así que la serología juega un papel clave en el diagnóstico de la enfermedad y correlacionarla con los eventos de abortos que están ocurriendo en un establecimiento. En un estudio realizado por colaboradores, se evaluaron tres diferentes kit comerciales de ELISA para detectar las seroprevalencias de un mismo establecimiento en el país. La concordancia entre los kits varió de débil a fuerte (coeficiente Kappa de Cohen = 0,58 a 0,83) pero a pesar de la imperfecta concordancia entre estos kits, el empleo de todos ellos permitió arribar a conclusiones similares respecto de la asociación estadísticamente significativa entre seropositividad a *N. caninum* y aborto (da Silva Silveira et al., 2020a). Cabe destacar que los kit comerciales utilizados en este trabajo son importados y de origen europeo (España, Holanda y Bélgica). En el mismo se menciona que el antígeno utilizado, en dos de los kit en la sensibilización de la placa es un lisado de taquizoitos de una cepa de referencia y el otro es una proteína recombinante. A esto se le suma el alto costo y los largos tiempos de espera de importación.

Como describimos en el tercer capítulo de esta tesis de doctorado, el aislamiento del parásito marca un hito a nivel país. En primer lugar porque se trata de las primeras cepas autóctonas de *N. caninum* que se logran aislar. Pero lo más interesante, es que el aislamiento y posterior caracterización genética de estas cuatro cepas permitió evidenciar la variabilidad que hay entre ellas. Si a este número reducido de cepas se le suman la caracterización genética de las diferentes muestras de abortos locales obtenemos una variabilidad mayor. Esa variabilidad se acentúa cuando las comparamos con las cepas de referencia (Nc-Liverpool y NC-1) de origen europeo y norteamericano. Obviamente como discutimos en la publicación, nuestros resultados son consistentes con la extensa variabilidad genética entre cepas que se observa en otras regiones. Por eso nos cuestionamos, si esta gran variabilidad que existe entre las cepas locales y las cepas de referencias (que son las utilizadas para generar los kit de diagnósticos comerciales) podrían afectar

la sensibilidad y especificidad de los kit comerciales de diagnóstico utilizados no solo a nivel local sino a nivel regional. Por tanto podemos concluir que existe la necesidad de desarrollar kits de diagnóstico locales que incluyan las cepas que circulan en cada región para ampliar la sensibilidad y especificidad de la técnica en cuestión. El desarrollo de un kit de diagnóstico serológico local no solo permitirá mejorar la detección del parásito sino también bajar los costos y tiempos de importación, lo cual podría redundar en la universalización del acceso al diagnóstico y permitir a los productores gestionar correctamente el manejo de su rodeo frente a la enfermedad. Creemos que el aislamiento de cepas constituye el primer hito hacia la generación de estos nuevos kit locales.

Un riguroso estudio reciente sobre la epidemiología de *N. caninum* en establecimientos lecheros en Uruguay (Macchi et al., 2020), confirma que la enfermedad sigue siendo endémica para el país, y que las cifras de seroprevalencia se mantienen muy similares a las encontradas en estudios realizados en el 2006 (Piaggio, 2006). Asimismo determina que la totalidad de los establecimientos tienen al menos un animal infectado, y que esa prevalencia se mantiene no solo con la transmisión vertical sino por transmisión horizontal. Este trabajo destaca además que los establecimientos con altos registros de seroprevalencia para el parásito son aquellos que poseen números crecientes de perros en los mismos (Macchi et al., 2020). Esto coincide con la detección del parasito como primer agente causante de abortos bovinos (Macías-Rioseco et al., 2020). A esto se le suma una publicación reciente sobre la seroprevalencia en perros en la ciudad de Montevideo donde se alcanza al 48%, definiéndose como una seroprevalencia alta (Satragno et al., 2020).

Por lo anteriormente mencionado, el diagnóstico eficaz de la enfermedad, el buen manejo y control del rodeo y las medidas relacionadas con el hospedero definitivo contribuyen a la disminución o eliminación del agente.

Debido a que el manejo y control del mismo es económicamente muy costoso y llevaría muchos años disminuir significativamente la incidencia del parásito en los establecimientos, la necesidad de desarrollar una vacuna constituye un consenso a nivel internacional (Horcajo et al., 2016). Es así que, utilizando diversas estrategias desde proteínas recombinantes, lisados de los parásitos hasta cepas atenuadas los esfuerzos a nivel mundial van en ese sentido (Bengoa-Luoni et al., 2019; Horcajo et al., 2016; Xu et al., 2019).

En este sentido el aislamiento de las nuevas cepas y su posterior caracterización fenotípica nos permitió evidenciar que las cuatro cepas aisladas presentan baja virulencia. Ésta, junto con otras características fenotípicas evaluadas en modelo de transmisión vertical, como por ejemplo la baja tasa de transmisión vertical y la baja potencialidad de generar abortos en la nueva progenie nos permitie proponerlas como cepas candidatas para una vacuna a cepa atenuada. Cabe destacar que nuestro grupo de investigación está realizando pruebas de CRISPR en otros parásitos, artículos publicados actualmente sobre este sistema de modificación genética en *N. caninum* dieron buenos resultados (Arranz-Solís et al., 2018). Con la posible cepa candidata y los estudios genéticos realizados sobre los posibles marcadores de virulencia y utilizando este sistema de modificación genética tendríamos todos los insumos para formular una vacuna contra el agente.

La caracterización genética de los nuevos aislamientos mediante marcadores moleculares, y posteriormente la caracterización fenotípica dejó en evidencia que los primeros no eran adecuados para correlacionarlos con el fenotipo descrito. Es así que nos propusimos abordar un estudio genético más exhaustivo de nuestras cepas para descifrar esta interrogante. Como

describimos en el capítulo V de esta tesis, la secuenciación de los nuevos aislados mediante nuevas tecnologías (Illumina, PacBio y Oxford Nanopore) nos permitió obtener nuevos resultados de los genomas de nuestras cepas locales y la cepa de referencia. Es así que con estos nuevos datos y un riguroso trabajo bioinformático de la Dra. Luisa Berna, logramos mejorar sustancialmente la anotación del genoma de *Neoespora caninum*. Este nuevo ensamblaje del genoma nos permitió romper con algunos paradigmas que eran aceptados por la comunidad científica. Hasta el momento se aceptaba que el genomas de *N. caninum* era altamente sintenico con el de *T. gondii*. El descubrimiento más significativo fue que *N. caninum* presenta 13 cromosomas, y no 14 como se pensaba hasta la fecha, y tiene importantes diferencias (reordenamiento cromosómicos) con el genoma de *T. gondii*. Finalmente, contamos con una lista más completa de nuevos genes anotados a partir del nuevo genoma, que permiten estudios de epítopes relevantes para diagnóstico o vacunas, o de diseño para atenuación de cepas (Berna et al., 2020).

En conclusión los conocimientos y productos generados en esta tesis de doctorado aportaron profundamente a la temática. Las áreas que se vieron favorecidas por la generación de resultados son varias, pero a nivel país especialmente el área epidemiológica y el desarrollo de métodos de diagnóstico son las que directamente tienen una aplicación en la producción, manejo e impacto económico sobre la enfermedad.

El aporte básico de conocimientos a nivel de la biología del parásito (genética y fenotipo) es fundamental para comprender la implicancia de la enfermedad.

7.0 Conclusiones

1- Se desarrollaron un conjunto de herramientas en el país para el abordaje de una nueva temática en el campo de la parasitología (cultivo de cepas y modelos de aislamiento).

2- Se optimizaron nuevas metodologías moleculares para la detección de *N. caninum* en diferentes muestras biológicas para complementar el certero diagnóstico de causales de aborto bovino.

3- Reportamos nuevos datos sobre la circulación y la epidemiología molecular de *N. caninum* en Uruguay.

4- Generamos el aislamiento de cuatro nuevas cepas autóctonas de *N. caninum*.

5- Caracterizamos genéticamente las cepas que circulan en el país y establecimos las relaciones genéticas con cepas regionales y mundiales, describiendo una nueva forma de agrupar genotipos.

6- Desarrollamos y optimizamos los diferentes modelos *"in vivo"* e *"in vitro"* en el país para la caracterización fenotípica del parásito.

7- Caracterizamos fenotípicamente las nuevas cepas aisladas generando el insumo para el diseño racional futuro una posible vacuna antigénicamente relevante para el país, y basada en el conocimiento de la genética particular de nuestras cepas.

8- Aportamos un valioso conjunto de datos sobre la composición y estructura del genoma de *N. caninum* y sus diferencias con el genoma de *T. gondii*.

9- Generamos artículos científicos de alta calidad aportando significativamente a la temática, y colaboramos con diferentes grupos de investigación del país y la region, contribuyendo asi con otros estudios relevantes.

8.0 Perspectivas

Los aislamientos de las cepas uruguayas y su caracterización genética y fenotípica nos permitirán generar nuevas preguntas y nuevos desafíos en el corto, mediano y largo plazo.

Como vimos en el transcurso de esta tesis, las cepas uruguayas de *N. caninum* difieren tanto genética como fenotípicamente de las cepas de referencias que actualmente se utilizan como base antigénica de los diferentes kit de diagnósticos. Una de las preguntas abiertas, que planificamos intentar abarcar en un futuro cercano es si los kit de diagnósticos importados nos estarán brindando un panorama certero de la enfermedad a nivel nacional. Existe la posibilidad de que las cepas locales difieran significativamente a nivel antigénico de las cepas de referencia. Por ende el diseño de un kit de diagnóstico nacional incluyendo las cepas autóctonas y su comparación con los kits comerciales importados es un desafío para el desarrollo de nuevas tecnologías.

Por otro lado un diagnóstico certero de la enfermedad nos permitiría aplicar un buen manejo y control del rodeo para la disminución o eliminación del agente. Debido a que el manejo y control del mismo es económicamente muy costoso y llevaría muchos años disminuir significativamente la incidencia del parásito en los establecimientos, la necesidad de desarrollar una vacuna constituye un consenso a nivel internacional. Es así que los esfuerzos a nivel mundial van en ese sentido, utilizando diversas estrategias desde proteínas recombinantes, lisados de los parásitos hasta cepas atenuadas. En este sentido, la identificación de una cepa avirulenta descrita en esta tesis de doctorado y los esfuerzos en pos de identificar las bases genéticas de estas características fenotípicas, será un insumo importante para la generación racional de nuevas cepas vacunales o la formulación de vacunas basadas en esta cepa.

Y por último, el conocimiento certero de la información genética asociada a los diversos fenotipos nos permitirá establecer líneas de investigación básica en la biología del parásito, como por ejemplo identificar los genes responsables de la virulencia, transmisión vertical, modulación del sistema inmune, entre otras áreas del conocimiento.

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Original Article
Livestock Diseases

Causes of abortion in dairy cows in Uruguay

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

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

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

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

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

[3] Programa de Posgrado en Salud Animal, Facultad de Veterinaria, Universidad de la Republica, Av. 18 de Julio 1824-1850, Montevideo, 11200, Uruguay.

[4] Unidad de Biología Molecular, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo, 11400, Uruguay.

[5] Laboratory of Apicomplexan Biology, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo, 11400, Uruguay.

[6] Laboratorio de Virología Molecular, Centro Universitario Regional Litoral Norte, Universidad de la República, Gral Fructuoso Rivera 1350, Salto, 50000, Uruguay.

[7] Unidad Mixta Pasteur, Instituto Nacional de Investigación Agropecuaria, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo, 11400, Uruguay.

[8] Laboratorio de Microbiología Molecular y Estructural, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo, 11400, Uruguay.

[9] División de Laboratorios Veterinarios, Ministerio de Ganadería, Agricultura y Pesca, Constituyente 1476, Montevideo, 11200, Uruguay.

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

TERMOS DE INDEXAÇÃO: Aborto bovino, bovinos de leite, Uruguai, *Campylobacter fetus*, *Coxiella burnetii*, *Neospora caninum*.

INTRODUCTION

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

In Chile, a longitudinal study of 20 years examined a total of 494 bovine fetuses from 270 farms. Forty-eight percent of cases were from dairy farms, 10.1% from beef farms, and the rest from mixed production farms. The etiology was determined in 59.7% of the cases, and 52.2% of the cases had an infectious cause. The most commonly identified agent was *Leptospira* spp. in 25.2%. About 22% of the cases had microscopic lesions compatible with *N. caninum*, and *B. abortus* was detected in 14.3%. The two main viral agents reported were bovine herpesvirus-1 in 13.4% and bovine viral diarrhea virus (BVDV) in 7.7% of the infectious cases (Meyer Zarzar 2013).

Infectious etiologies are among the most commonly reported abortigenic agents in cattle in Uruguay. A study conducted from 2002 to 2004 analyzed 431 aborted bovine fetuses from dairy (54%) and beef (46%) farms. In 41% of the cases with diagnosis, the abortion was attributed to leptospirosis based on the detection of leptospiral maternal antibodies, and *N. caninum* was identified in 36%. *Campylobacter fetus* was reported in 13% of the cases, one of which, based on microscopic observation, was determined as coinfected with *Tritrichomonas foetus*. The bacterial etiology with the lowest reported frequency was *B. abortus* in 3% of the cases. Lastly, about 23% of the examined fetuses did not have any macroscopic or microscopic lesions and were of undetermined cause (Easton 2006).

The diagnosis of bovine abortion is complex, and the diversity of causes cannot be identified despite extensive laboratory testing (Antoniassi et al. 2013, Clothier & Anderson 2016). Bovine abortion has been considered a syndrome because of the complexity of their causes (Anderson et al. 1990, Campero et al. 2003). Fetal and placental autolysis is common, precludes the observation of lesions interfering with the successful identification of infectious agents. The circulation of more than one abortigenic agent in the same herd could represent another difficulty in the diagnosis of bovine abortion, particularly in epidemic outbreaks (Macías-Rioseco et al. 2019a). Likewise, bacterial cultures from fetal tissues and placentas are frequently contaminated with non-pathogenic or opportunistic bacteria (Clothier & Anderson 2016), making interpretation of results difficult, particularly in cases without lesions typical of bacterial infection. Nonetheless, a plethora of diagnostic approaches such as necropsy, histology, immunohistochemistry, bacteriology, virology, immunology and molecular biology assays, aid in the diagnosis of bovine abortion.

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

MATERIALS AND METHODS

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

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

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

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

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

Additionally, abomasal fluid or placenta smears were examined directly under dark field microscopy to assess for agents morphologically compatible with *Campylobacter* spp., *Leptospira* spp. and *Tritrichomonas foetus*. PCR for bovine herpesvirus-1 (BHV-1) and RT-PCR for BVDV were performed in pools of liver, lung, spleen, kidney, thymus, heart, brain, placenta, lymph node, and adrenal gland obtained individually in all cases that included fetuses. For BVDV, the primers V324mod (5'- ATGCCCWTAGTAGGACTAGCA-3') and V326mod (5' -WCAACTCCATGTGCCATGTAC-3') were used based on Maya et al. (2016). Briefly, the mix was exposed at 95°C for 10 min, then 45 cycles of 10 sec at 95°C, for denaturation, and 60 sec at 50°C for annealing and extension. The primers gCBoHV F (5'-GCGGGGGCTCGCCGAGGA-3') and gCBoHV R (5'-GGAGCGCACGGTCAGGGGC-3') were used for BHV-1 PCR. The mix was exposed at 95°C for 5min, then 35 cycles of 30 sec at 95°C, for denaturation, and 30 sec at 60°C for annealing, 1 min at 72°C for extension and lastly, 10 min at 72°C (Silva et al. 2007).

The IHC for BVDV antigen detection was done only in cases with a positive RT-PCR result for this virus. Heat induced antigen retrieval was performed in a decloaking chamber (Biocare Medical). A mouse IgG anti-BVDV (VMRD, Pullman/WA) was applied as primary antibody, followed by anti-mouse IgG horseradish peroxidase (HRP)-labeled polymer (DAKO, Santa Clara/CA), and 3-amino-9-ethylcarbazole (AEC, DAKO, Santa Clara/CA) as the chromogen. In one fetus with pneumonia with syncytial cells, RT-PCR for bovine parainfluenza-3 (BPI-3) virus was done from frozen samples of lung and the amplification products were sequenced, as we have previously described for this same case (Macías-Rioseco et al. 2019b).

Based on the presence of compatible lesions (necrotizing and suppurative placentitis with intratrophoblastic bacteria), IHC for *C. burnetii* and *Chlamydia* spp. were done in selected cases (Dilbeck & McElwain 1994, Giannitti et al. 2016). When a positive result for *C. burnetii* by IHC was obtained, PCR was used for detection of this pathogen in the formalin-fixed paraffin-embedded (FFPE) blocks containing placenta (Lorenz et al. 1998). The IHC procedures for *Chlamydia* spp. and *C. burnetii* and the PCR in FFPE placenta in these same cases has recently been published by our group (Macías-Rioseco et al. 2019c).

Immunohistochemistry and PCR for detection of *N. caninum* antigen and DNA, respectively, were done only in cases with compatible histologic lesions in the brain, heart, placenta and/or liver. For the IHC, a goat polyclonal antibody (VMRD, Pullman, WA) against *N. caninum* was used as a primary antibody, anti-goat IgG horseradish peroxidase (HRP)-labeled polymer (Vector polymer enzyme detection kit, Burlingame/CA) as the secondary antibody, and 3-amino-9-ethylcarbazole as the chromogen (DAKO, Santa Clara/CA). The PCR for *N. caninum* was done following the

procedure described by Yamage et al. (1996). PCR products were visualized on 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, USA), purified with QIAquick PCR Purification Kit and sequenced in house at the sequencing service of the "Institut Pasteur de Montevideo", Uruguay. All tests were performed with appropriate positive and negative controls for each run.

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

RESULTS

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

The etiology of the abortion was determined in 54 (53%) cases, while the cause was undetermined in 48 (47%). Of the cases with undetermined etiology, 11 (23%) had inflammatory and/or necrotizing lesions in various tissues suggesting an infectious process, although no agent could be identified in the tissues by the set of diagnostic tests performed in the study. Of the 54 cases that had an etiologic diagnosis, 51 (94.4%) were caused by infectious agents. Thirty-eight of the 51 (74.5%) were caused by agents that are primarily recognized as reproductive pathogens, one was caused by BPI-3 virus and another by *Salmonella enterica* serovar Newport. The remainder 11 cases (21.5%) were associated with opportunistic bacteria. The 38 cases caused by reproductive pathogens included *N. caninum* (30 cases), *C. burnetii* (6 cases), and *C. fetus* subsp. *venerealis* (2 cases). Of the 11 cases caused by opportunistic pathogens, *Escherichia coli* was identified in 4 cases, *Streptococcus* spp. in 2 cases, and *Streptococcus pyogenes*, *Staphylococcus* sp., *Trueperella pyogenes*, *Providencia stuartii*, and *Mannheimia* sp. were identified in one case each. Placentitis, bronchopneumonia, and/or hepatitis were the main histologic lesions observed in association with these opportunistic agents. Regarding the three cases with non-infectious causes (5.6% of the 54 cases with an identified cause), in two cases the death of the fetuses was due to dystocia and in one case the abortion was caused by a congenital mesothelioma affecting the abdominal and pelvic viscera that was extensive enough to be considered incompatible with life. The percentage of diagnosis was variable according to the material sent to the laboratory. When only the fetus was sent, the diagnosis was made in 50.9% of the cases; when the fetus and placenta were received the diagnosis was made in 62.8% of the cases, and when only the placenta was available the diagnosis was made in 37.7% of the cases (Table 1).

The 30 abortions caused by *N. caninum* (55.6% of the 54 cases with determined etiology) were from different dairy farms, accounting for 53.3% of the 45 farms included in the study. Of the six abortions caused by *C. burnetii*, five were from the same farm. *Campylobacter fetus* subsp. *venerealis* was the etiology in 2 cases from different farms.

The number of cases submitted per farm varied from one to 28, and the number of causes of abortion diagnosed on each farm varied from one to four (Fig.1). In farm 42, four different etiologic agents causing abortion were identified: *N. caninum* in two cases, *C. burnetii* in five, *Salmonella enterica* serovar Newport in one, and BPI-3 virus in another. In farm 43, five aborted fetuses were sent to the laboratory during an abortion outbreak, one abortion was caused by *C. fetus* subsp. *venerealis* and two by *N. caninum*; in the other two fetuses the cause of abortion was undetermined (Macías-Rioseco et al. 2019a). In farm 1, one abortion was caused by *N. caninum*, another by *E. coli*, and one was due to dystocia. In farm 17, an etiologic diagnosis could only be confirmed in one of nine examined cases.

The cases caused by *N. caninum* corresponded to abortions within the second trimester of gestation. The 30 fetuses aborted by neosporosis had typical lesions including non-suppurative encephalitis and gliosis (29 fetuses), myocarditis (24), myositis (diaphragm or tongue) (20) hepatitis (15), interstitial nephritis (10) and/or interstitial pneumonia (6). Additionally, placenta was submitted along with the fetus in 10 of these 30 cases, 7 of which had placentitis. The six abortions caused by *C. burnetii* corresponded to full-term gestations and had moderate to severe multifocal necrotizing and suppurative placentitis with intralesional and intratrophoblastic bacteria, and only one case had mild neutrophilic alveolitis. No cases of coxiellosis were diagnosed in cases where no placenta was available for examination. One case of abortion due to *C. fetus* subsp. *venerealis* had a non-suppurative fibrinous epicarditis and myocarditis; the placenta was not available for examination. The other case of *C. fetus* subsp. *venerealis* abortion had suppurative placentitis with arteriolitis and fibrinoid necrosis, neutrophilic bronchiolitis and alveolitis along with neutrophilic and histiocytic portal hepatitis. Suppurative and necrotizing placentitis was observed in cases associated with *E. coli*, *T. pyogenes*, and *S. enterica* serovar Newport. The latter also had intralesional coccobacilli in the placenta, along with minimal to mild neutrophilic lymphadenitis. In one case caused by *Staphylococcus* sp., the agent was isolated from the skin and from abomasal fluid, the fetus had diffuse hyperkeratosis, neutrophilic, histiocytic and fibrinous synovitis, along with moderate non-suppurative interstitial pneumonia

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

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

with neutrophilic alveolitis and mild non-suppurative meningoencephalitis. One case caused by *Mannheimia* sp. had a moderate lymphohistiocytic and neutrophilic placentitis with multifocal trophoblastic necrosis. BPI-3 virus caused multifocal neutrophilic and histiocytic alveolitis (pneumonia) with a moderate number of syncytial cells in the lungs and intestines in one case (Macías-Rioseco et al. 2019b). In cases with lesions but without an identified etiology, the lesions consisted of myocarditis, myositis, glossitis, cerebral gliosis, nephritis, pneumonia, and hepatitis.

Brucella abortus was not isolated in any case. Other bacterial agents such as *Acinetobacter lwofii*, *Aerococcus urinae*, *Providencia* sp., *Yersinia* sp., *E. coli*, *Enterobacter* sp., *Corynebacterium* sp. and *Serratia* sp. were isolated on bacterial cultures, but due the absence of associated lesions expected for bacterial infections, these abortions were classified as of undetermined etiology. The gene lipL32 of *Leptospira* spp. was detected in one sample of liver by PCR, the causality of the abortion was not attributed to this agent based on the lack of fetal lesions generally associated with leptospirosis. None of the tested fetal pericardial and/or thoracic fluids were reactive at the cutoff reference point for MAT for *Leptospira* spp. antibodies, including the case that was PCR-positive for this agent. *Leptospira* spp. culture was negative in all cases. PCR for BHV-1 was negative in all cases. RT-PCR for BVDV was positive in three cases; two of them were in co-infection with *N. caninum*, and the protozoon was determined as the causal agent based on the presence of typical lesions and the positive results for *N. caninum* PCR and IHC. Due the absence of lesions, the etiology on the third BVDV-positive case was categorized as undetermined. Two of the three BVDV PCR-positive fetuses were aborted in the second trimester and the other in the third trimester of gestation. To assess whether these fetuses congenitally infected with BVDV harbored high antigenic viral loads and thus were persistently infected, IHC for BVDV antigen detection was performed in several tissues (brain, lung, heart, thymus, liver and small intestine), with negative results in all three cases (data not shown). Based on these results, we interpreted that the fetuses were suffering from transient BVDV infections.

DISCUSSION

In this case series, the most common cause of abortion was *N. caninum*. A previous study in Uruguay, showed a seroprevalence of *N. caninum* of 22% in dairy cows and 92% of the herds (Piaggio 2006). Our series was composed mainly of fetuses of gestational age in the second trimester, which may be explained in part by the high frequency of neosporosis. Abortions due to *N. caninum* are most commonly seen during the second and third trimesters of gestation (McAllister 2016). Our results and the high proportion of farms with seropositive cattle to *N. caninum* suggest that abortions caused by this agent potentially occur in most farms (Silveira 2019).

Coxiella burnetii is rarely reported as a cause of abortion in cattle (Agerholm 2014). In our study, this agent was observed as a sporadic cause of abortion (only one case in one dairy farm) and as a cluster of five cases within a period of five months in another farm (Macías-Rioseco et al. 2019c). We were able to identify abortions due to coxiellosis based on the microscopic

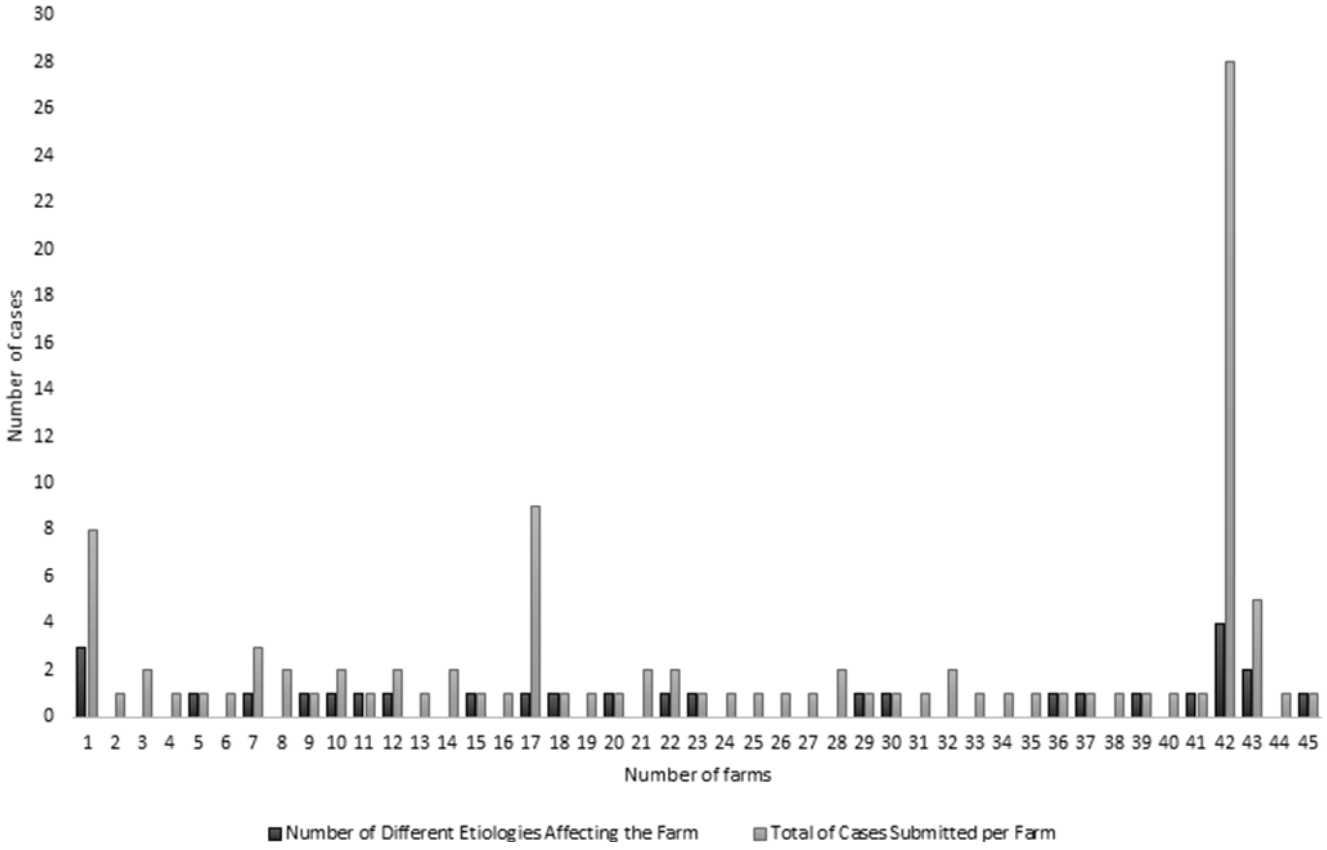

Fig.1. Frequency of different etiologies affecting each farm and total number of cases submitted per farm.

evaluation of the placenta followed by IHC along with PCR for *C. burnetii*. In all cases, the diagnosis of coxiellosis was based on the presence of necrotizing and suppurative placentitis with intralesional and intratrophoblastic bacteria identified as *C. burnetii*. The presence of lesions associated with the bacteria is key to the diagnosis of coxiellosis (Bildfell et al. 2000, Hazlett et al. 2013) since *C. burnetti* can be identified by PCR in the placenta of ruminants without being the cause of abortion (Hazlett et al. 2013).

These results suggest that *C. burnetii* is a previously undiagnosed cause of abortion in dairy cattle in Uruguay and highlight the importance of examining the placenta in aborted cattle to achieve this diagnosis. The presence of antibodies against *C. burnetii* in slaughterhouse workers from Uruguay has been associated with history of clinical signs, and a clinical case of endocarditis (Moreira et al. 1987), showing that this agent is an occupational hazard for veterinarians and slaughterhouse workers. Also, antibodies against *C. burnetii* have been found in different animal species in this country, including cattle (Moreira et al. 1987).

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

As seen in this study, several causes of abortion were diagnosed in some farms, which indicates that the diagnosis of a primary cause of abortion, including neosporosis, campylobacteriosis, or coxiellosis, does not exclude other causes of abortion. Hence, it is recommended to attempt the diagnosis in as many cases as possible in order to increase the chances of detecting other abortifacients and better understand the situation of each farm.

In our case series, only one of the tested fetuses was positive for *Leptospira* spp. by PCR on a liver sample Sequencing confirmed that the infection corresponded to the species *L. interrogans*. Fetal pericardial and/or thoracic fluids were antibody-negative by MAT in all fetuses, including the PCR-positive one. DIF and/or dark field microscopy for the detection of leptospires were also negative in all fetal imprints and abomasal fluids. While *Leptospira* spp. cannot be excluded as a relevant abortigenic pathogen in cattle in Uruguay, our study did not allow for confirmation of *Leptospira* spp. as a cause of abortions following the diagnostic criteria and case definition we established. Many *Leptospira* species and serovars have recently been isolated from bovine urine and blood samples in Uruguay, including *L. interrogans* serogroup Pomona serovar Kennewicki (20 strains), *L. interrogans* serogroup Canicola serovar Canicola (1 strain), *L. borgpetersenii* serogroup Sejroe serovar Hardjo (10 strains) and *L. noguchii* (9 strains, belonging to a variety of serogroups) (Zarantonelli et al. 2018). According to that study, 20% of the almost 1,000 sampled cows were eliminating *Leptospira* spp. in the urine (Zarantonelli et al. 2018). It is striking that with such a high number of animals eliminating leptospires in the urine, the present study did not find abortions caused by leptospirosis. It cannot be ruled out that *Leptospira*-induced abortions might occur with no detectable infection of the fetus or placenta and be caused by other mechanisms including the pathogen-triggered inflammatory cascade (Raghupathy 2001, Zi et al. 2015). Examples of such abortigenic scenarios include subclinical infections in the mother due to urinary tract infection (Schieve et al. 1994), periodontitis (Zi et al. 2015), bacterial vaginosis (Giakoumelou et al. 2016), among other pathologies. In animals such inflammatory processes linked to premature birth or miscarriage have also been described e.g. in bovine viral diarrhea (Moennig & Liess 1995), suggesting that different pathogen strains can produce different clinical outcomes. The role of the different *Leptospira* species found in the urine of healthy cattle as a cause of sporadic abortions and other reproductive failures should thus be further investigated (Zarantonelli et al. 2018). In the present series of abortions, most cases corresponded to sporadic abortions and only few outbreaks were studied. It is possible that infections by some *Leptospira* species and/or specific serovars may cause abortion outbreaks in herds, which probably were not subjected to confirmatory laboratory investigation during the period of this study. Establishing a surveillance system to study abortion outbreaks in Uruguay seems important to better understand the pathogenesis, epidemiology and best diagnostic techniques for leptospirosis and other abortive diseases in the country.

Viral abortions in cattle are reported in low frequencies in several studies (Vaucher et al. 2011, Clothier & Anderson 2016). In this case series, one abortion was caused by BPI-3 virus. This agent had been previously isolated from a bovine fetus and the case diagnosed in our study has been published elsewhere (Macías-Rioseco et al. 2019b). Abortions due to BHV-1 and BVDV were not observed in this series, although BVDV RNA was detected in three fetuses. None of the three cases had lesions compatible to BVDV, and IHC reactivities for BVDV antigen in liver, small intestine and brain were negative. The molecular detection of the pathogen confirms the circulation of the virus in these herds. Even though it seems that BVDV was not responsible for the abortions in these cases, it is important to test affected herds to identify persistently infected animals and the possibility of the occurrence of other forms of BVDV-associated diseases in the farms. Two of the positive BVDV animals were also positive for *N. caninum* by PCR and/or IHC and in fact had tissue lesions consistent with neosporosis, that was considered the primary cause of the abortions. It has been suggested that BVDV infections allow other pathogens to easily cross the fetoplacental barrier, increasing the risk of abortion (Murray 1991, Quinn et al. 2004).

One abortion in our series was due to *Salmonella* Newport, which is rarely reported causing abortions (Campero et al. 2018). In this farm the serovar Newport also caused neonatal calf diarrhea and neonatal mortality due to septicemia during the same period (data not shown), indicating that the abortion was part of the spectrum of diseases typically associated with salmonellosis in dairy cattle and not an isolated event. In cattle, *Salmonella enterica* serovars Typhimurium, Dublin, and Newport are the most commonly cause of salmonellosis.

The clinical disease can be enteritic and/or septicemic, the latter can result in abortion in pregnant cattle (Uzal et al. 2016, Campero et al. 2018, Costa et al. 2018). Abortions due to *Salmonella* are mostly associated with *Salmonella* Dublin (Campero et al. 2003), and less frequently, with *S*. Typhimurium (Easton 2006).

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

CONCLUSIONS

Neosporosis is the main cause of abortions in the dairy cattle population that we studied.

Coxiellosis is a cause of outbreaks or sporadic cases of abortions in Uruguay.

Campylobacteriosis is still a cause of abortions, and most likely infertility in dairy cattle in the country.

It is important to further investigate the pathogenesis mechanisms, epidemiology and diagnosis of leptospirosis to determine the importance of this disease as a cause of abortion in Uruguay.

The systematic diagnosis of bovine abortion is necessary to set possible strategies of prevention and control, besides monitoring and surveillance of reproductive diseases in dairy cattle, some of which can represent a risk to public health.

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