# Tesis de Doctorado en Biología

## (PEDECIBA)

***Caracterización genética, antigénica y patogénica del virus de la enfermedad infecciosa de la bursa (IBDV) en Uruguay***

**Mag. Gonzalo Tomás**

**Diciembre, 2019**

Tesis para la obtención del título de Doctor en Ciencias Biológicas del Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Subárea Genética, realizada en la Facultad de Ciencias de la Universidad de la República (UdelaR), Uruguay.

Título:

Caracterización genética, antigénica y patogénica del virus de la enfermedad infecciosa de la bursa (IBDV) en Uruguay.

Autor:

Mag. Gonzalo Tomás

Orientadores:

Dr. Ruben Pérez, Sección Genética Evolutiva, Facultad de Ciencias, UdelaR.

Dr. Ariel Vagnozzi, Laboratorio de Aves, Instituto de Virología, INTA-Castelar.

Tribunal:

Dr. Juan Arbiza

Dra. Silvia Llambí

Dr. Ali Saadoun

# RESUMEN

El virus de la enfermedad infecciosa de la bursa (IBDV) es uno de los patógenos más problemáticos en la industria avícola mundial debido a que afecta severamente el sistema inmune de las aves y provoca grandes pérdidas económicas. El nivel de daño provocado por el virus depende de varios factores entre los que se destacan la edad del ave, su estado inmune, y el tipo de cepa involucrado. El virus posee un genoma de ARN doble hebra bisegmentado que favorece el surgimiento de variantes virales. Desde su primer reporte, se han descrito varias cepas con características genéticas, antigénicas y patogénicas diferentes. Esta Tesis presenta la descripción y posterior caracterización de un tipo de cepa particular que se denominó *distinct*. Esta cepa es la más frecuentemente encontrada en Uruguay y la región. Inicialmente se realizó la caracterización genética parcial de esta cepa analizando fragmentos de ambos segmentos genómicos. Esto incluyó un análisis exhaustivo de la base de datos de secuencias que evidenció la presencia de cepas *distinct* en varios continentes desde hace más de 30 años. Distintos tipos de análisis genéticos determinaron que las cepas *distinct* son en realidad un grupo viral genéticamente diferente del resto de cepas tradicionalmente conocidas. Complementariamente se obtuvo y analizó el primer genoma completo de una cepa *distinct*. Posteriormente se desarrolló un método de detección específico de las cepas *distinct* basado en PCR en tiempo real. El diseño del método incluyó el análisis detallado de las secuencias de IBDV disponibles con el fin de obtener un ensayo eficiente y robusto. La validación realizada fue exitosa y la metodología presentó un excelente desempeño. A continuación se estudió la historia epidemiológica de las cepas *distinct* a través de aproximaciones filodinámicas y

filogeográficas utilizando inferencias Bayesianas. Los resultados obtenidos sugieren que las cepas *distinct* son uno de los grupos virales más antiguos, habiéndose originado prácticamente al mismo tiempo que las cepas involucradas en el primer brote de la enfermedad. Además, se determinó el lugar de origen más probable de estas cepas en Europa del Este (Polonia y Hungría), y las rutas migratorias que habrían dado lugar a la diseminación de las cepas *distinct* por varios continentes. Finalmente se estudiaron las características antigénicas de una cepa *distinct* uruguaya, así como sus efectos en infecciones experimentales de aves. Los resultados indicaron que la cepa es antigénicamente diferente a las cepas tradicionales, y que si bien no provoca signos clínicos en las aves infectadas, afecta la bursa de Fabricio generando un cuadro de inmunodepresión severo.

En conjunto, el trabajo realizado durante esta Tesis permitió establecer la existencia de una cepa viral previamente desconocida y profundizar en la descripción de sus características genéticas, antigénicas y patogénicas, así como en aspectos de su evolución e historia epidemiológica.

# ABSTRACT

Infectious bursal disease virus is one of the most problematic pathogens in the poultry industry, producing mayor economic losses through the impairment of the birds' immune system. Several factors influence the level of damage caused by the virus, mainly the bird's age, its immune system status, and the viral strain involved. The emergence of viral variants is favored by the nature of the virus genome, composed by double strand, bisegmented RNA molecules. Since the first report of the disease, strains with different genetic, antigenic and pathogenic characteristics were described. This Thesis is on the first description and characterization of a particular strain here denoted as *distinct*. This is the most frequent strain in Uruguay and the region. Firstly, the partial genome characterization was done though the analysis of partial fragments of both genome segments. This included an exhaustive analysis of the sequence database which resulted in the finding of *distinct* strains collected in various continents since more than 30 years ago. Different genetic approaches determined that the *distinct* strains compose a group of genetically different strains compared to traditional strains. Additionally, the first complete genome of a *distinct* strain was obtained and analyzed. Later, it was developed a qPCR-based method for the specific detection of the *distinct* strains. The design of the method was based on the genetic differences previously detected in order to achieve the development of an efficient and robust assay. This study included the validation of the method, which allowed the demonstration of the correct performance of the assay. Then, the epidemiological history of the *distinct* strains was analyzed through phylodinamic and phylogeographic approaches using Bayesian inferences. This study suggests that the *distinct* strains would be one of the most

ancestral viral groups, emerging almost at the same time that the strains involved in the first outbreak of the disease. Also, the most probable origin location, Eastern Europe (Poland and Hungary), and the migration routes that triggered the intercontinental spread of the strain were estimated. Finally, the antigenic characteristics of a Uruguayan *distinct* strain were analyzed, as well as the consequences of the infection of SPF birds with the same strain. These analyses revealed that the Uruguayan *distinct* strain is antigenically different from the rest of traditional strains, and that it produces bursal atrophy and a severe immunosuppression in the absence of any clinical sign.

Overall, this Thesis evidences the existence of a previously neglected viral strain and provides a deep description of its genetic, antigenic and pathogenic characteristics, as well as shedding light over its evolution and epidemiological history.

# INTRODUCCIÓN GENERAL

## SURGIMIENTO DE LA ENFERMEDAD INFECCIOSA DE LA BURSA

La Enfermedad Infecciosa de la Bursa (IBD, por sus siglas en inglés) se detectó por primera vez en el otoño de 1957 en una granja de pollos de engorde ubicada en las cercanías de la comunidad de Gumboro, Delaware, EEUU (Cosgrove, 1962), debido a lo cual también se la conoce como Enfermedad de Gumboro (Fig. 1). En este reporte se la describió como una enfermedad que afecta principalmente aves jóvenes, provocando diarrea, anorexia, depresión, postración y temblores; su diseminación fue muy rápida e infectó los galpones cercanos en pocos días con una mortalidad de hasta 10%.

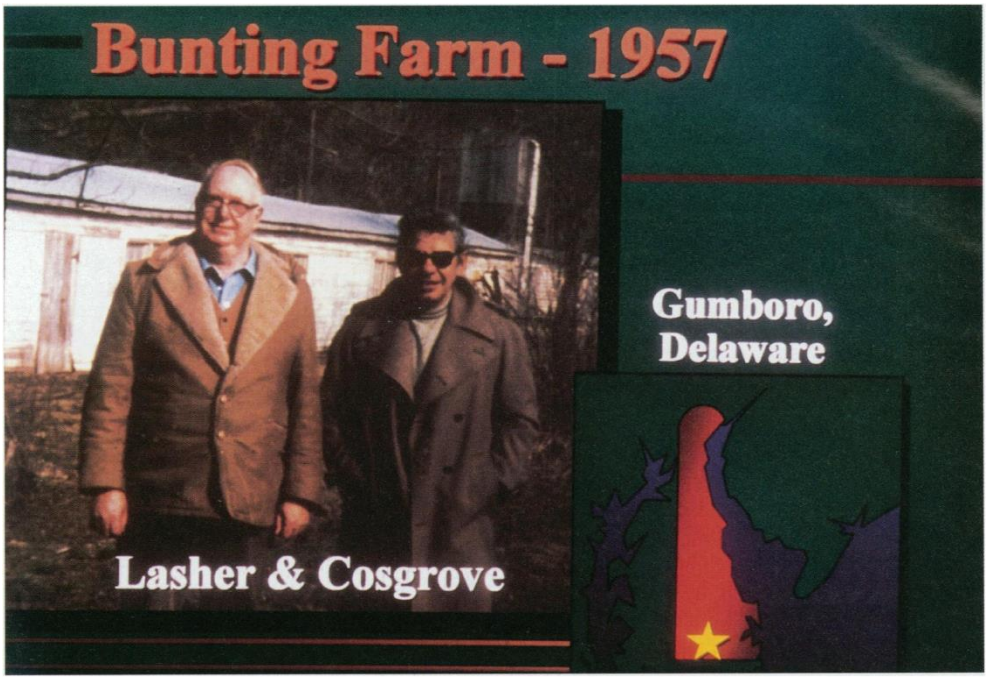


Figura 1- Foto tomada en la primera granja donde se observó la enfermedad infecciosa de la bursa, llamada Bunting Farm, en las cercanías de la comunidad de

Gumboro, Delaware. Aparecen el Dr. Albert Cosgrove (derecha) y el Dr. Hiram Lasher (izquierda). Tomado de Lasher & Davis (1997).

Inicialmente se la llamó 'nefrosis aviar', debido a que frecuentemente se observaban lesiones graves en los riñones de las aves afectadas, e incluso se pensaba que el agente etiológico era una cepa nefropatógena del virus de la bronquitis infecciosa aviar. Luego se determinó que en la mayoría de los casos observados inicialmente existía una coinfección entre el agente etiológico de la enfermedad infecciosa de la bursa y una cepa nefropatógena del virus de la bronquitis infecciosa aviar, lo cual llevó a la confusión inicial y complicó la identificación del verdadero agente etiológico (Winterfield & Hitchner, 1962).

El primer aislamiento del virus de Gumboro (*Infectious Bursal Disease Virus,* IBDV) fue realizado por un grupo encabezado por el Dr. Winterfield en 1962 (Winterfield *et al.*, 1962). Fue así que unos años más tarde, a partir de la realización de estudios que involucraron la infección de aves bajo condiciones controladas, se vio que el virus provocaba lesiones graves principalmente en la bursa de Fabricio, órgano linfoide primario de las aves (Cho & Edgar, 1969; Hitchner, 1970). Luego se determinó que el daño en la bursa de Fabricio generaba inmunodepresión, incrementando la susceptibilidad a patógenos oportunistas normalmente presentes en los establecimientos avícolas (Allan *et al.*, 1972; Faragher *et al.*, 1974).

En 1980, el grupo encabezado por McFerran describió la existencia de un serotipo diferente aislado de pavos provenientes de Irlanda del Norte (McFerran *et al.*, 1980). Poco tiempo después, el mismo tipo de virus fue descrito en EEUU, y se sugirió llamarlo 'serotipo 2' para diferenciarlo del resto de los virus identificados, ahora llamados del 'serotipo 1' (Jackwood & Saif, 1983; McNulty & Saif, 1988). Infecciones experimentales con virus del

serotipo 2 demostraron que éstos no provocaban enfermedad en los pollos (Ismail *et al.*, 1988a). Si bien en un principio se pensó que los virus del serotipo 2 podían ser candidatos a utilizarse como vacunas, se vio que no existía una adecuada protección cruzada entre los dos serotipos (Chettle & Wyeth, 1989).

La enfermedad se controló exitosamente mediante el uso de vacunas inactivadas y atenuadas hasta los años 1980s, cuando resurgió en la península de Delmarva, una región compartida entre los estados de Delaware, Maryland y Virginia. Este resurgimiento se debió a la aparición de cepas virales antigénicamente diferentes, las cuales se denominaron variantes antigénicas o simplemente variantes para diferenciarlas de las que circulaban previamente (cepas "clásicas"). Las cepas variantes se caracterizaron por generar una fuerte inmunodepresión temprana, incluso en aves con altos niveles de anticuerpos maternales contra cepas clásicas (Saif, 1984; Rosenberger & Cloud, 1985; Rosenberger *et al.*, 1985).

Unos pocos años más tarde, aparecieron en Europa brotes de IBDV que causaban grandes daños y altas tasas de mortalidad (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). Las cepas involucradas en estos brotes se denominaron hipervirulentas y no presentaban cambios antigénicos significativos con las cepas clásicas, pero sí un incremento notorio en su patogenicidad.

## VIRUS DE LA ENFERMEDAD INFECCIOSA DE LA BURSA

### *TAXONOMÍA*

Según la clasificación taxonómica actual, IBDV es la única especie conocida del género *Avibirnavirus* dentro de la familia *Birnaviridae* (Delmas *et al.*, 2019). Esta familia está compuesta por virus desnudos con cápside de

simetría icosaédrica, número de triangulación T=13, de unos 70 nm de diámetro (Böttcher *et al.*, 1997; Coulibaly *et al.*, 2005) (Fig. 2).

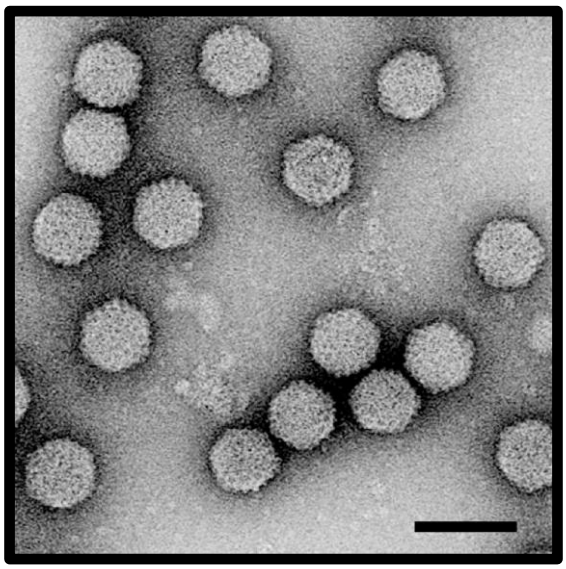

Figura 2- Micrografía electrónica de partículas virales purificadas de IBDV. La barra corresponde a 100 nm. Modificado de Castón *et al.* (2001).

Su genoma, compuesto por dos segmentos de ARN doble hebra, es característico de todos los birnavirus y da nombre a la familia; "bi" por el carácter bisegmentado y "rna" por la naturaleza del material genético (Nick *et al.*, 1976). Además del género *Avibirnavirus* se han descrito otros tres géneros dentro de esta familia: *Aquabirnavirus*, *Blosnavirus* y *Entomobirnavirus*. Dentro del género *Aquabirnavirus* se encuentran tres especies que infectan animales acuáticos como son el *infectious pancreatic necrosis virus* que infecta peces salmónidos, el *Tellina virus* que infecta moluscos bivalvos y el *Yellowtail ascites virus* que infecta peces del género *Seriola*.

El género *Blosnavirus* está formado únicamente por la especie *Blotched snakehead virus* el cual fue originalmente aislado de una línea celular

derivada del pez *Channa lucius.* Finalmente, el género *Entomobirnavirus* contiene la especie *Drosophila X virus* de la mosca de la fruta como único miembro actual.

*GENOMA*

Los segmentos que forman el genoma de IBDV se denominan A y B. El segmento genómico A (3,2 Kpb) posee dos marcos abiertos de lectura (ORF) parcialmente solapados, denominados ORF-A1 y ORF-A2 (Kibenge *et al.*, 1990) (Fig. 3). La región correspondiente al ORF-A1 codifica la proteína VP5 y tiene entre 438 y 450 nt de longitud dependiendo de la cepa viral (Mundt *et al.*, 1995). El ORF-A2 es de 3039 nt y codifica una poliproteína que, mediante un proceso de clivaje en el que participan componentes virales y del hospedero, genera las proteínas VP2, VP3, VP4, y cuatro péptidos pequeños (Sánchez & Rodriguez, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002). Las regiones no codificantes (UTR) de los extremos 5' y 3' se extienden por 96 y 95 nt respectivamente. El segmento genómico B (2,8 Kpb) codifica en un único ORF de 2640 nt la proteína VP1 (Spies *et al.*, 1987). Las UTR de los extremos 5' y 3' tienen un largo de 111 y 82 nt respectivamente (Mundt & Müller, 1995).

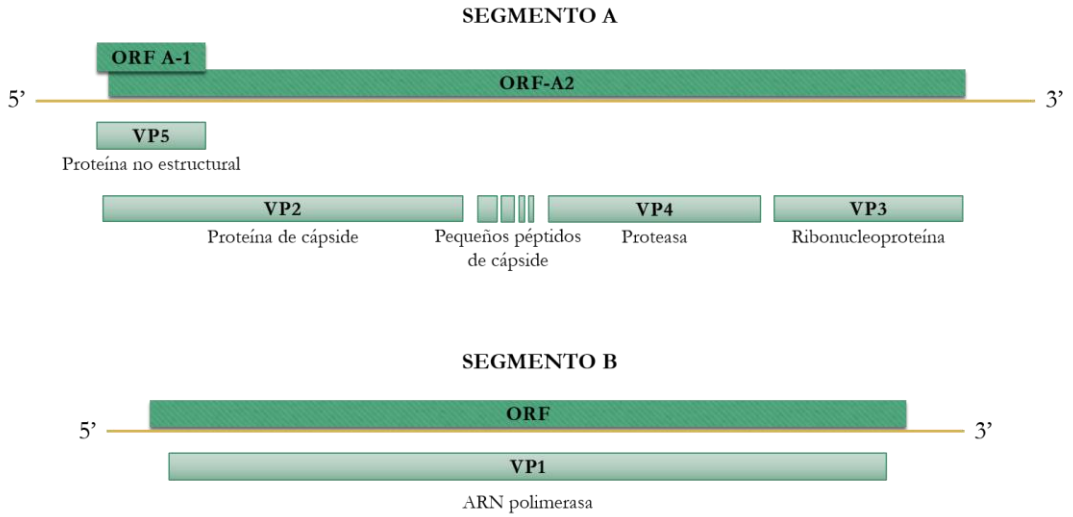


Figura 3- Esquema representando los dos segmentos genómicos de IBDV y sus productos proteicos correspondientes.

*PROTEÍNAS VIRALES*

La proteína VP1 es una RNA polimerasa dependiente de RNA encargada de replicar el genoma y transcribir los RNA mensajeros. VP1 también funciona como proteína cebadora mediante la utilización de residuos de guanina que son incorporados de forma covalente por un proceso de auto guanililación (Pan *et al.*, 2009). Los residuos de guanina unidos a VP1 hibridan con residuos de citosina localizados en los extremos 3' en ambos segmentos genómicos para actuar como cebadores. VP1 tiene tres regiones principales: región N-terminal (1-167 aa), a la cual se le atribuye la función cebadora; región central (168-658), donde se encuentran todos los dominios responsables de la actividad polimerasa (dedos, pulgar y palma) y región C-terminal (659-878 aa), la cual funcionaría evitando errores en el proceso de cebado al inicio de la polimerización (*back-priming*).

La proteína VP2 es la principal componente de la cápside viral, la cual está formada por 260 trímeros de esta proteína (Coulibaly *et al.*, 2005; Saugar *et*

*al.*, 2005). Estructuralmente se divide en tres dominios denominados B (*base*), S (*shell*) y P (*projection*) (Garriga *et al.*, 2006; Lee *et al.*, 2006) (Fig. 4A). Los dominios B y S son relativamente conservados y están formados por los extremos N- y C-terminal, mientras que el dominio P es central e incluye una región hipervariable ubicada entre los residuos 206 y 350 (Bayliss *et al.*, 1990). En esta región hipervariable de VP2 se encuentran cuatro bucles que se proyectan hacia el exterior, denominados $P_{BC}$, $P_{DE}$, $P_{FG}$ y $P_{HI}$, para conformar la región más expuesta de la partícula viral (Letzel *et al.*, 2007) (Fig. 4A y B). Los residuos de estos bucles intervienen en el tropismo celular, en la virulencia y en la antigenicidad (Brandt *et al.*, 2001; Jackwood *et al.*, 2008; Qi *et al.*, 2009, 2013; Noor *et al.*, 2014). La proteína VP2 se genera por un proceso de maduración del precursor pVP2 derivado del clivaje de la poliproteína (Sánchez & Rodriguez, 1999). El procesamiento de pVP2 también produce los péptidos pequeños pep46, pep7a, pep7b y pep11 (Da Costa *et al.*, 2002). Algunos de estos péptidos pequeños estarían involucrados en procesos importantes del ciclo viral, tales como el ensamblaje de la partícula viral y la desestabilización de la membrana celular para permitir el ingreso del virus (Chevalier *et al.*, 2005; Galloux *et al.*, 2007).

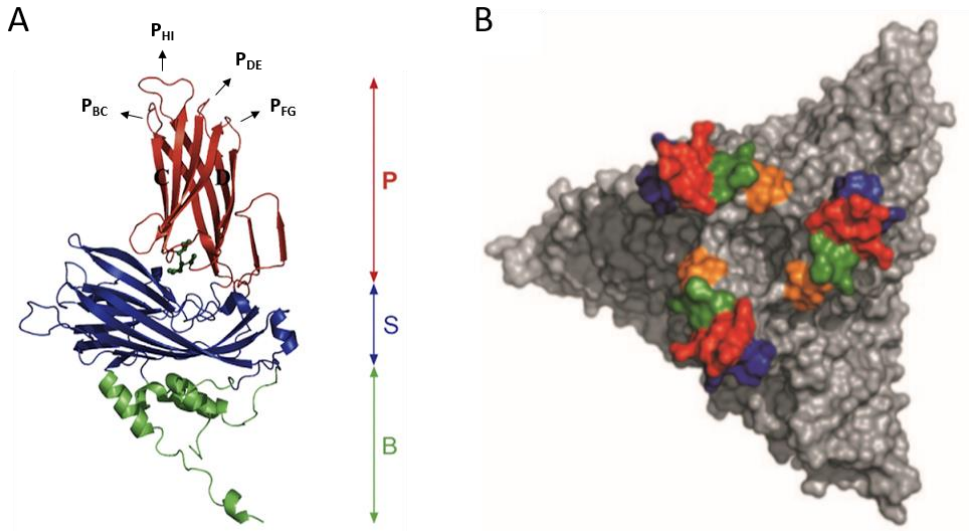


Figura 4- Representación esquemática de la proteína VP2. (A) Monómero de VP2. Los dominios P, S y B se muestran en diferente color. Se indica la posición de cada uno de los bucles $P_{BC}$, $P_{DE}$, $P_{FG}$ y $P_{HI}$. (B) Trímero de VP2. Se muestran los bucles correspondientes a cada monómero en colores diferentes ($P_{BC}$, azul; $P_{DE}$, verde; $P_{FG}$, amarillo; $P_{HI}$, rojo). Modificado de Letzel *et al.* (2007) y Delgui *et al.* (2009).

VP3 es una proteína multifunción. La interacción del extremo C-terminal de VP3 con el extremo C-terminal de pVP2 es importante para la correcta formación de la cápside viral, por lo cual se ha descrito a VP3 como una proteína *scaffold* fundamental durante la morfogénesis (Maraver *et al.*, 2003; Oña *et al.*, 2004; Saugar *et al.*, 2005; Mata *et al.*, 2018). Durante el ensamblado de la cápside, VP3 interacciona con la polimerasa viral VP1 y funciona como regulador de la actividad transcripcional (Lombardo *et al.*, 1999). VP3 también se une a ambos segmentos genómicos para formar complejos ribonucleoproteicos (Luque *et al.*, 2009).

La proteína VP4 es una proteasa viral encargada del procesamiento de la poliproteína (Birghan, 2000; Lejal *et al.*, 2000). Específicamente, VP4 es una serín-proteasa donde los residuos Ser-652 y Lys-692 funcionan como díada catalítica (Lejal *et al.*, 2000). A VP4 también se le atribuye un rol importante

en la inhibición de la respuesta inmune del hospedero, ya que se ha visto que inhibe la expresión de interferón tipo I a través de su interacción con la proteína GILZ (*glucocorticoid-induced leucine zipper*) (He *et al.*, 2018).

La proteína no estructural VP5, codificada por el ORF-A1 del segmento A, es una proteína transmembrana de clase II. Esta proteína no es esencial para la replicación viral, pero cumple un rol importante en la salida del virus de la célula (Rosenberger & Gelb, 1978; Mundt *et al.*, 1997; Lombardo *et al.*, 2000; Wu *et al.*, 2009). En etapas tempranas de la infección VP5 actúa inhibiendo la apoptosis celular, posiblemente favoreciendo la producción de más progenie viral (Liu & Vakharia, 2006; Wei *et al.*, 2011). Contrariamente, en etapas tardías de la infección actúa promoviendo la apoptosis y favoreciendo la diseminación del virus (Li *et al.*, 2012).

### TRANSMISIÓN VIRAL

La transmisión del virus ocurre de forma horizontal. A partir de las 48 hs post infección, las aves infectadas excretan partículas virales a través de las fecas (Takase *et al.*, 1982; Zhao *et al.*, 2013). Una vez que las fecas se secan, las partículas virales se diseminan por el aire y pueden contaminar galpones adyacentes. No se ha detectado excreción viral a través de las vías respiratorias de las aves infectadas (Zhao *et al.*, 2013). La resistencia del virus en el ambiente favorece su transmisión y permanencia en los galpones, habiendo estudios que demuestran la persistencia del virus hasta por 122 días en el ambiente (Benton *et al.*, 1967; Zhao *et al.*, 2013).

El virus ha sido aislado de diversas especies de aves silvestres que funcionan como reservorio y contribuirían con la dispersión del virus. Entre esas especies se encuentran patos, gansos, gorriones e incluso pingüinos

(Jackwood *et al.*, 2005; Wang *et al.*, 2007). Además, la presencia de anticuerpos anti-IBDV también se detecta en otras especies de aves acuáticas y no acuáticas (Ogawa *et al.*, 1998; Fagbohun *et al.*, 2000; Hollmén *et al.*, 2000).

## REPLICACIÓN VIRAL

El ingreso del virus a la célula comienza con la interacción entre la proteína de cápside VP2 y los receptores celulares. Se han identificado diferentes receptores celulares, tales como la proteína de choque térmico cHSP90 (Lin *et al.*, 2007), la α4β1 integrina (Delgui *et al.*, 2009), y la inmunoglobulina M de superficie sIgM (Luo *et al.*, 2010). La entrada a la célula se realiza por la vía endosomal, específicamente por macropinocitosis (Gimenez *et al.*, 2015) (Fig. 5).

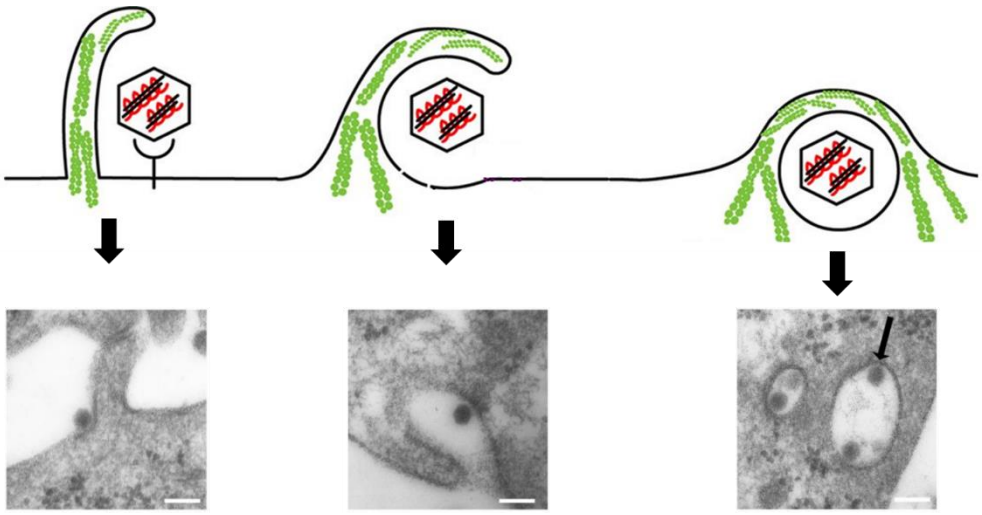

Figura 5- Modo de ingreso de IBDV a la célula por macropinocitosis. Arriba: representación esquemática del ingreso de IBDV a la célula por el mecanismo de macropinocitosis. Abajo: micrografías electrónicas donde se muestran imágenes reales de las etapas esquematizadas. Modificado de Gimenez *et al.* (2015, 2018).

Una vez que el virus se localiza dentro de la célula, es transportado hacia endosomas tempranos donde la disminución de la concentración de calcio promovería la liberación del pep46, que hasta ese momento permanecía formando parte de la cápside, e induce la formación de poros en la membrana del endosoma (Galloux *et al.*, 2007). El tamaño de los poros (~10 nm) no permite la salida del virus (~60 nm) del endosoma, pero sí el intercambio de pequeñas moléculas necesarias para iniciar la transcripción. En los birnavirus, la generación de los transcriptos de ARN ocurre dentro de la partícula viral intacta, para migrar posteriormente al citoplasma donde son traducidos.

El proceso de ensamblado comienza con el procesamiento de la poliproteína para liberar las proteínas pVP2 y VP3, mecanismo mediado por la proteasa viral VP4. Posteriormente, la proteína VP3 y la proteasa celular PurSA compiten para interactuar con pVP2 (Irigoyen *et al.*, 2012) (Fig. 6).

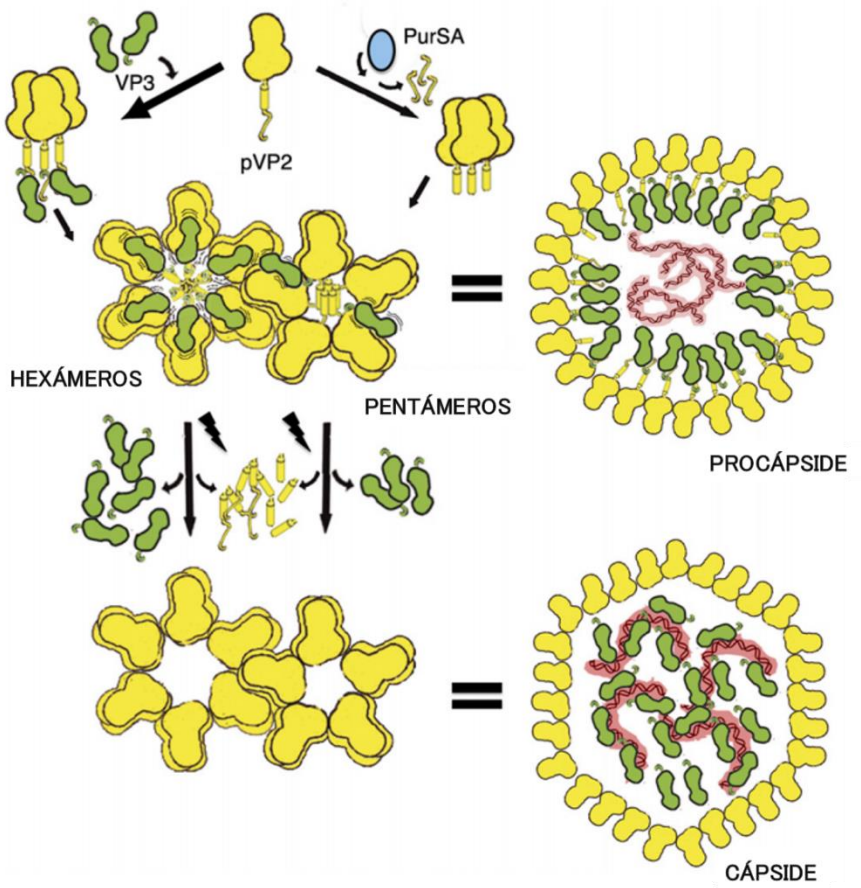


Figura 6- Modelo del ensamblado de la cápside de IBDV. El precursor de la cápside, pVP2, interactúa con PurSA (azul) o VP3 (verde), para formar pentámeros o hexámeros, respectivamente. La interacción de ambas estructuras forma la procápside con el genoma en su interior (rojo). La posterior acción autoprotelítica de pVP2 dispara la formación de la cápside madura, en donde VP3 se mantiene asociada al genoma viral. Modificado de Irigoyen *et al.* (2012).

Cuando VP3 se une a pVP2, impide que PurSA procese a esta última, lo cual favorece la formación de hexámeros. Por el contrario, si pVP2 es procesada por PurSA, la proteína de cápside forma pentámeros. Dado que los pentámeros de pVP2 también unen algunas proteínas VP3, la interacción entre las VP3 de los hexámeros y pentámeros forma una estructura llamada procápside. Esta estructura contiene el genoma viral con VP1 unido en sus

extremos 5' e interactuando también con VP3. Finalmente, la propia pVP2 es autoclivada liberando a VP3 y formando la partícula viral madura.

En la etapa final del ciclo, la acción promotora de apoptosis de la proteína VP5 permite la ruptura de la membrana celular y la liberación de las partículas virales (Li et al., 2012).

### PATOGÉNESIS E INMUNODEPRESIÓN

La inmunodepresión asociada a IBDV se debe a que el principal órgano afectado es la bursa de Fabricio, órgano linfoide primario exclusivo de las aves en donde ocurre la maduración de los linfocitos B (Cooper *et al.*, 1966). IBDV replica en los linfocitos B inmaduros provocando una depleción linfocitaria, principal causa de la inmunodepresión. Las aves inmunodeprimidas son más susceptibles a la infección con patógenos oportunistas frecuentemente encontrados en las granjas, como el virus de la anemia infecciosa, virus de la bronquitis infecciosa, u otros patógenos bacterianos del sistema respiratorio y gastrointestinal (Rosenberger & Cloud, 1989; Nader *et al.*, 2013). Además, las aves inmunodeprimidas también muestran una respuesta reducida a la aplicación de vacunas, lo que aumenta aún más la problemática (Giambrone, 1979).

La forma más comúnmente asociada con el ingreso del virus es la vía oral, aunque también ingresa por inhalación (Zhao *et al.*, 2013), no existiendo reportes de transmisión vertical. El período de incubación dura entre 2 y 3 días, dependiendo principalmente de la virulencia de la cepa (Van Den Berg *et al.*, 2000). Cuando el virus llega al intestino, ocurre la primera replicación en macrófagos del duodeno, yeyuno y ciego (Müller *et al.*, 1979). A través del sistema venoso portal llegan al hígado dentro de las 5 horas post-infección (hpi). A través del torrente sanguíneo principal el virus alcanza otros órganos,

entre ellos la bursa de Fabricio, en cuyos folículos se encuentran las principales células blanco, los linfocitos B inmaduros. A las 13 hpi, la mayoría de los folículos son positivos para la presencia del virus, y a las 16 hpi ocurre una nueva viremia masiva asociada a una segunda replicación en otros órganos linfoides, tales como timo, bazo, tonsilas cecales y glándula de Harder.

La replicación del virus en los linfocitos B inmaduros de la bursa de Fabricio está asociada con la infiltración de linfocitos T CD4+ y CD8+ activados en este órgano (Poonia & Charan, 2004). Algunas evidencias sugieren que los linfocitos T restringen la replicación de IBDV en etapas tempranas de la infección (Rautenschlein *et al.*, 2002). Sin embargo, la acción de estas células a través de la liberación de diferentes citoquinas conduce a la activación de los macrófagos, quienes a su vez producen otros factores proinflamatorios. Esto genera una tormenta de citoquinas y una activación exacerbada de la respuesta inmune, conduciendo a la intensificación de la destrucción del tejido bursal y el retardo de su recuperación (Sharma *et al.*, 2000). Por otro lado, se ha visto que la apoptosis juega un rol importante en la patogenia desencadenada por IBDV, tanto en células infectadas como no infectadas (Jungmann *et al.*, 2001).

Una vez que el virus desaparece de las aves, estas se recuperan y los linfocitos B comienzan a repoblar los folículos de la bursa de Fabricio (Kim *et al.*, 1999; Withers *et al.*, 2006). Las aves recuperadas no logran recomponer por completo la respuesta humoral y sufren de niveles variables de inmunodepresión. Un factor que incide directamente en el nivel de inmunodepresión es la edad del ave, en donde las aves más jóvenes muestran menor capacidad de recomposición del sistema inmune humoral. Otro factor influyente es la virulencia de la cepa de IBDV, estando las cepas

más virulentas asociadas con una menor recuperación (Allan *et al.*, 1972; Higashihara *et al.*, 1991).

**SIGNOS CLÍNICOS**

El período de incubación de la enfermedad es corto, observándose los primeros signos clínicos entre 2 y 3 días luego de la exposición al virus. Entre los signos más típicos se encuentran la diarrea blanquecina, asociada con una tendencia de algunas aves a picotear su propia cloaca, el erizamiento de las plumas, la disminución en el consumo de alimento y agua, y niveles variables de letargia sin reacción a estímulos externos (Cosgrove, 1962) (Fig. 7). La presencia de signos clínicos y la gravedad de los mismos dependen de varios factores, estando entre los más importantes la edad de las aves, el estado inmune de las mismas, su línea genética, y la virulencia de la cepa viral involucrada (Fadly & Nazerian, 1983; Chettle *et al.*, 1985; Bumstead *et al.*, 1993).

La edad a la cual las aves son más susceptibles a la ocurrencia de signos clínicos es en el período comprendido entre las 3 y 6 semanas de vida. Esto se ha relacionado directamente con el nivel de desarrollo de la bursa de Fabricio, ya que en este período es cuando el órgano alcanza su mayor estado de maduración y el mayor número de linfocitos B inmaduros (Ismail *et al.*, 1987). Las aves infectadas de forma temprana, previo a la semana 3, generalmente no muestran signos clínicos pero sufren de una severa inmunodepresión (Faragher *et al.*, 1974). Aves de 6 semanas son menos susceptibles a la infección y por lo tanto disminuye la ocurrencia de manifestaciones clínicas tras la exposición al virus, aunque hay reportes de la enfermedad clínica en aves de 14 y 15 semanas de edad (Ley *et al.*, 1979).

La disminución de los signos clínicos se debe a que este órgano naturalmente se atrofia y deja de ser funcional hacia la madurez sexual de las aves, alrededor de la semana 18-20 de edad (Ciriaco *et al.*, 2003). En aves bursectomizadas el virus no es capaz de replicarse (Hiraga *et al.*, 1994).

El estado inmune de las aves es otro factor importante, principalmente la presencia de anticuerpos maternos. Las aves jóvenes que tienen niveles altos de anticuerpos maternos son menos susceptibles a la enfermedad, siempre y cuando exista un buen nivel de homología entre los anticuerpos neutralizantes maternos y la cepa viral circulante (Ide *et al.*, 1978).

Figura 7- Ave afectada por la enfermedad infecciosa de la bursa. Se aprecia el erizamiento de las plumas y cierto nivel de letargia en comparación con las aves circundantes. Fuente: https://fieldcasestudy.com.

La línea genética del ave hospedero es también un factor determinante en la aparición o no de signos clínicos y en su severidad. Un estudio reciente realizado con aves SPF de dos tipos, uno correspondiente a pollos de engorde (Plymouth Rock) y otro correspondiente a aves de postura (White Leghorn), obtuvo resultados que sustentan esta hipótesis (Silva *et al.*, 2016). En base a los signos clínicos, nivel de mortalidad y análisis histopatológicos de tejidos linfoide y renal, las aves de postura mostraron una mayor susceptibilidad a los efectos patológicos provocados por la infección con una cepa hipervirulenta. Resultados similares se obtuvieron utilizando una cepa clásica como virus desafío y comparando la infección de aves de postura y pollos de engorde (Nielsen *et al.*, 1998). Esta respuesta diferencial observada entre aves de postura y pollos de engorde parece deberse a diferencias en la modulación de los niveles de la respuesta inmune innata, específicamente en cuanto a las células T y los niveles de expresión de distintas citoquinas (Tippenhauer *et al.*, 2013).

Por último, el tipo de cepa involucrado en la infección también es relevante a la hora de la generación de signos clínicos, tema profundizado en la siguiente sección.

### DIVERSIDAD VIRAL

El genoma de ARN doble hebra de IBDV está sometido a altas tasas de mutación de entre $10^{-5}$ y $10^{-6}$ mutaciones/sitio/generación (Peck & Lauring, 2018). Esto se refleja en tasas de sustitución que rondan las $10^{-4}$ sustituciones/sitio/año (Silva *et al.*, 2013; Alfonso-Morales *et al.*, 2015). Sumado a la capacidad de variar por mutación, IBDV puede también generar variantes por recombinación y reordenamiento de sus segmentos genómicos

(Wei *et al.*, 2006; He *et al.*, 2009; Soubies *et al.*, 2017; Pikuła *et al.*, 2018). Esta capacidad de variar su genoma ha fomentado el surgimiento y establecimiento de poblaciones virales genéticamente heterogéneas que pueden variar antigénica y patogénicamente.

Los virus del serotipo 2 se han aislado principalmente de pavos, especie en donde predominan según relevamientos serológicos (Jackwood *et al.*, 1982; Chin *et al.*, 1984). Aunque este serotipo infectan pavos y pollos, lo cual se evidencia por la seroconversión de las aves infectadas, no generan signos clínicos ni daños en la bursa de Fabricio o alteraciones en la respuesta inmune, por lo cual se los considera no patogénicos (Perelman & Heller, 1983; Jackwood *et al.*, 1984; Ismail *et al.*, 1988b).

Dentro de los virus considerados patogénicos pertenecientes al serotipo 1 se encuentran las primeras cepas aisladas de IBDV, hoy conocidas como clásicas virulentas. Estas cepas inicialmente se asociaron con brotes donde la mortalidad específica rara vez superó el 10 % (Cosgrove, 1962; Parkhurst, 1964). En ensayos de laboratorio bajo condiciones controladas, infectando aves de postura de tipo White Leghorn SPF de entre 3 y 6 semanas de edad, se observaron niveles de mortalidad de entre 10% y 50%, dependiendo de la cepa utilizada (Eterradossi & Saif, 2013).

Un grupo viral muy cercanamente emparentado con las cepas clásicas virulentas, aunque con características genéticas y patogénicas diferentes, son las denominadas cepas clásicas atenuadas. Este grupo, como su nombre lo sugiere, está casi exclusivamente compuesto por cepas de origen vacunal, además de cepas adaptadas a cultivos celulares y embriones de pollo. Genéticamente hay una clara diferenciación de estas cepas y las cepas clásicas virulentas, ubicándose en clados diferentes y encontrándose marcadores específicos a nivel aminoacídico en la región hipervariable de

VP2 (Dormitorio *et al.*, 1997). Patogénicamente también se observan diferencias, ya que las clásicas atenuadas no producen mortalidad en las aves, aunque pueden generar daño variable en la bursa de Fabricio y distintos niveles de inmunodepresión (Giambrone & Clay, 1986; Mazariegos *et al.*, 1990).

Las cepas variantes o variantes antigénicas son otro importante grupo viral claramente diferente del resto de las cepas. Ensayos *in vitro* de neutralización cruzada evidenciaron la existencia de diferencias antigénicas mayores entre las cepas variantes y vacunas de tipo clásica (Ismail & Saif, 1988). Posteriormente se demostró mediante estudios *in vivo* de protección cruzada que las cepas vacunales clásicas no generan niveles de protección adecuados contra desafíos con cepas variantes (Ismail *et al.*, 1990). Patogénicamente las cepas variantes se caracterizan por generar una severa atrofia de la bursa de Fabricio e inmunodepresión en ausencia de signos clínicos (Eterradossi & Saif, 2013) (Fig. 8).

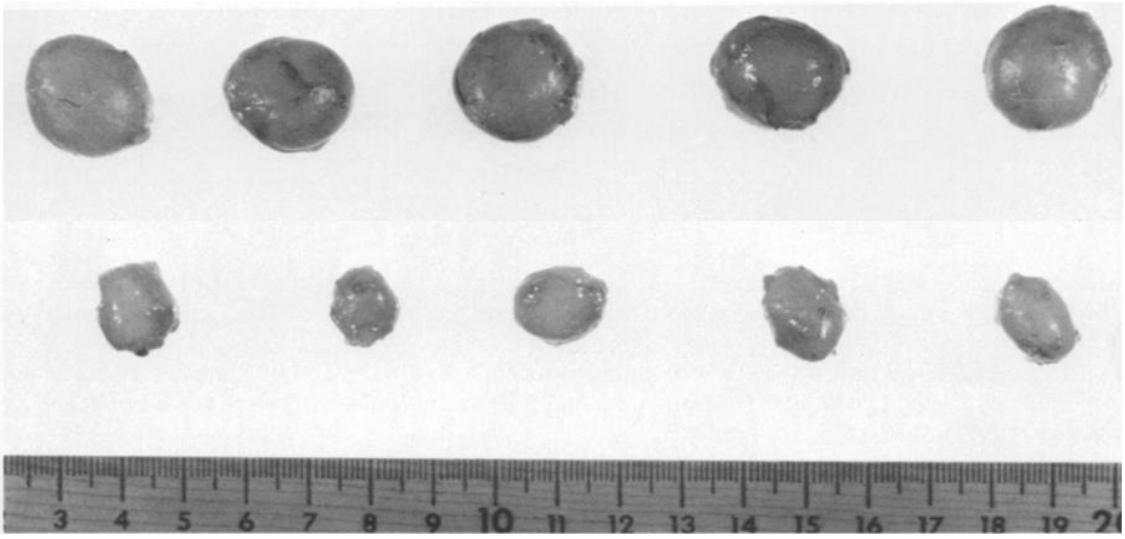

Figura 8- Bursas de Fabricius de aves SPF de 5 semanas de edad colectadas 5 días post infección. Arriba: bursas de Fabricius pertenecientes a aves sin infectar (control sin infección); abajo: bursas de Fabricius de aves infectadas con la cepa variante IN. Tomado y modificado de Ismail *et al.* (1990).

Las cepas hipervirulentas son una de las más problemáticas, debido a su amplia distribución y prevalencia en varios continentes y al daño provocado en las aves infectadas. Se caracterizan por ser muy patogénicas, causando niveles de mortalidad que alcanzan el 25% en pollos parrilleros y 60 % en aves de postura (van den Berg *et al.*, 1991; Nunoya *et al.*, 1992). Estudios bajo condiciones controladas, utilizando aves ponedoras SPF, detectan tasas de mortalidad de entre 50% y 100% (Eterradossi & Saif, 2013). A diferencia de lo que ocurre con las cepas variantes, las vacunas realizadas en base a cepas clásicas son eficaces para proteger a las aves frente a desafíos con cepas hipervirulentas (van den Berg *et al.*, 1991; Eterradossi *et al.*, 1992). Sin embargo, se evidencian diferencias a nivel antigénico entre ambas cepas cuando se analiza el patrón de afinidad con un panel de anticuerpos monoclonales (Eterradossi *et al.*, 1997).

Otro grupo viral diferente es el conformado por las cepas de IBDV presentes en Australia. En ese país no se observan en campo casos clínicos típicos de infección por IBDV, aunque las cepas australianas generan inmunodepresión en ausencia de signos clínicos (Firth, 1974; Westbury, 1978; MacKenzie & Spradbrow, 1983). Los IBDV australianos se subdividen en dos grupos de cepas, llamadas clásicas y variantes, que difieren tanto en aspectos genéticos como antigénicos entre sí y con las cepas clásicas, variantes e hipervirulentas encontradas en otros continentes (Sapats & Ignjatovic, 2000; Ignjatovic & Sapats, 2002). Hasta el día de hoy, en Australia únicamente se reportan casos de cepas de IBDV locales.

**MÉTODOS DE CLASIFICACIÓN Y CARACTERIZACIÓN**

La aproximación más utilizada actualmente para clasificar las cepas de IBDV es a través de análisis genéticos, específicamente de la región hipervariable de VP2 (Wu *et al.*, 2007). Esta es una región genómica pequeña (~400 nt), fácilmente amplificable por PCR, y que permite diferenciar las cepas de IBDV mediante su comparación filogenética. Además, el análisis de su secuencia permite identificar marcadores aminoacídicos relacionados con la patogenicidad, antigenicidad, capacidad de crecimiento en cultivos celulares, y la identificación de cepas (Vakharia *et al.*, 1994; Brandt *et al.*, 2001; Letzel *et al.*, 2007).

Además de analizar la región hipervariable de VP2, la cual se encuentra en el segmento genómico A, también es aconsejable estudiar el segmento genómico B, principalmente por la ocurrencia de reordenamientos genómicos. Existen numerosos reportes de cepas de IBDV con segmentos genómicos reordenados, la mayoría de los cuales involucra cepas hipervirulentas y cepas clásicas atenuadas aunque también se han visto otras combinaciones, incluyendo reordenamientos con virus del serotipo 2 (Le Nouën *et al.*, 2006; Wei *et al.*, 2008; Chen *et al.*, 2012; Kasanga *et al.*, 2013; Lu *et al.*, 2015; Soubies *et al.*, 2017, 2018; Pikuła *et al.*, 2018; Wang *et al.*, 2019).

Desde el punto de vista antigénico, se han utilizado diferentes estrategias para clasificar y caracterizar cepas de IBDV. Una de las más utilizadas son los ensayos de AC-ELISA con diferentes paneles de anticuerpos monoclonales (Snyder *et al.*, 1992; Eterradossi *et al.*, 1997). Según la reacción con los diferentes anticuerpos, los virus se clasifican en grupos antigénicos que se corresponden con cepas de referencia clásicas, variantes e hipervirulentas. Los anticuerpos monoclonales utilizados en estos ensayos se unen a epítopes específicos de VP2. El análisis de la relación entre la afinidad de los

anticuerpos monoclonales con los cambios aminoacídicos en la región hipervariable de VP2, permitió identificar cuatro tramos de importancia crítica llamados picos hidrofílicos (Vakharia *et al.*, 1994). Estos picos se corresponden con los cuatro bucles encontrados en la parte más expuesta de la proteína VP2 ($P_{BC}$, $P_{DE}$, $P_{FG}$, $P_{HI}$). Cambios aminoacídicos en esas regiones están directamente relacionados con la disminución de afinidad por ciertos anticuerpos monoclonales (Letzel *et al.*, 2007).

Otra forma de clasificar y caracterizar una cepa de IBDV es a través de estudios de patogenicidad mediante la infección de aves de postura SPF. Este tipo de ensayo no se realiza de forma rutinaria debido a la complejidad del procedimiento, incluyendo la disponibilidad de aves SPF y de instalaciones de biocontención adecuadas. Para comparar resultados obtenidos en estos ensayos, es importante considerar la edad de las aves infectadas, la línea genética utilizada, la dosis y vías de administración del virus desafío, y la posible presencia de agentes contaminantes en el inóculo. Este tipo de ensayos de patogenicidad permitirían diferenciar infecciones provocadas por cepas clásicas virulentas, variantes e hipervirulentas, aunque los límites de esta clasificación no están completamente definidos (Eterradossi & Saif, 2013).

## ESTRATEGIAS DE CONTROL

Debido a su amplia distribución y a la gran resistencia del virus a diferentes condiciones ambientales y desinfectantes, IBDV es considerado ubicuo en la industria avícola (Benton *et al.*, 1967; Petek *et al.*, 1973; Meulemans & Halen, 1982). Las estrategias de control apuntan a reducir la carga de virus de campo presente en los galpones, empleando estrictos manejos biosanitario y realizando una correcta administración de vacunas.

Las vacunas vivas atenuadas son muy utilizadas para el control de IBDV. Estas vacunas se desarrollan mediante la atenuación de cepas de campo por pasajes seriados en embriones de pollo SPF o cultivos celulares (Müller *et al.*, 2012). Dependiendo del grado de atenuación, las vacunas se clasifican en suaves, intermedias, e intermedias plus o calientes (Organización Mundial de Sanidad Animal, 2018). Las vacunas suaves son típicamente usadas para generar una respuesta inmune primaria en aves reproductoras de alrededor de 8 semanas de edad, previo a la aplicación de vacunas inactivadas (Skeeles *et al.*, 1979). Se caracterizan por no producir daño en la bursa de Fabricio, pero su eficiencia es muy pobre frente a la presencia de anticuerpos maternos. Las vacunas intermedias e intermedias plus se utilizan en pollos parrilleros y aves de postura, aunque también suelen utilizarse en aves reproductoras jóvenes si estas están bajo riesgo de infección con una cepa de campo patogénica. Estas vacunas pueden aplicarse según diferentes esquemas de vacunación, aunque generalmente se aplican dos dosis, una cuando las aves tienen entre 10-14 días de edad y otra entre 7-10 días más tarde. Dado que los anticuerpos maternos circulantes en los pollos jóvenes interfieren con la vacunación, es importante hacer un seguimiento de la caída de anticuerpos maternos para establecer el mejor momento de aplicación de la vacuna (Eterradossi & Saif, 2013).

Complementariamente al uso de vacunas vivas atenuadas, el control de IBDV suele incluir la utilización de vacunas inactivadas en aves reproductoras. Estas vacunas se aplican para generar niveles de anticuerpos altos, uniformes y duraderos, previo a que las aves alcancen la edad productiva, entre las 16 y 20 semanas de edad. De esta manera, las aves transfieren niveles altos de anticuerpos a los pollos jóvenes, principalmente a través de la yema, para protegerlos de posibles desafíos de campo durante las primeras 2 a 3 semanas de vida (Wyeth & Cullen, 1978; Fahey *et al.*, 1987).

Otro tipo de vacunas desarrolladas desde hace varios años están basadas en el uso de vectores virales como el herpesvirus del pavo, virus de Newcastle, fowlpox virus, o virus de la enfermedad de Marek, que expresan la proteína VP2 de IBDV (Heine & Boyle, 1993; Darteil *et al.*, 1995; Tsukamoto *et al.*, 1999; Huang *et al.*, 2004). Una ventaja de este tipo de vacunas es que pueden aplicarse *in ovo* en embriones de 18 días de edad o en pollitos de 1 día de edad, sin interferir con los anticuerpos maternos (Bublot *et al.*, 2007).

Alternativamente, se han desarrollado vacunas basadas en inmunocomplejos antígeno-anticuerpo que consisten en una mezcla de anticuerpos anti-IBDV y un virus vacunal (Whitfill *et al.*, 1995). Al igual que las vacunas recombinantes, las vacunas de tipo antígeno-anticuerpo son efectivas tras su aplicación en embriones de 18 días y pollitos de 1 día de edad, además de que no se detecta interferencia con los anticuerpos maternos (Haddad *et al.*, 1997; Giambrone *et al.*, 2001; Iván *et al.*, 2005).

A pesar del mejoramiento en el diseño y efectividad de las vacunas, siguen existiendo problemas en el control de IBDV. Estos problemas son generados por diversos factores, incluyendo errores en el manejo y la aplicación de las vacunas, y la emergencia de nuevas variantes virales. Por esta razón, es importante conocer el tipo de cepas de campo que circula en los sistemas productivos, ya que esto permite el diseño de estrategias de control específicas y eficaces.

# HIPÓTESIS DE TRABAJO

IBDV es un virus con genoma de ARN bisegmentado, y por lo tanto tiene un gran potencial para generar variantes genéticas con nuevas características antigénicas y patogénicas. Caracterizar estas variantes, así como conocer su historia epidemiológica, es una herramienta imprescindible para comprender la evolución viral y generar información epidemiológica que mejore las estrategias de control de este relevante patógeno aviar.

# OBJETIVOS

### OBJETIVO GENERAL

Avanzar en el conocimiento de las cepas de IBDV más frecuentes en la industria avícola uruguaya.

### OBJETIVOS ESPECÍFICOS

- Determinar las principales características genéticas de las cepas de IBDV que circulan actualmente en la industria avícola uruguaya.
- Diseñar, estandarizar y validar una metodología basada en PCR en tiempo real para la identificación específica de las cepas de IBDV uruguayas.
- Analizar el pasado epidemiológico de las cepas de IBDV uruguayas a través de estudios filodinámicos y filogeográficos.
- Conocer las características antigénicas y patogénicas de las cepas de IBDV circulantes en Uruguay.

# CAPÍTULO 1- PRIMERA DESCRIPCIÓN DE LAS CEPAS DISTINCT

En este primer capítulo se presenta la descripción de las cepas *distinct* como un grupo viral de circulación global, ancestral, y con características genéticas particulares. Parte de los resultados aquí presentados, específicamente aquellos relacionados con la primera descripción de las cepas *distinct* en Uruguay, se obtuvieron en coautoría con otros integrantes del grupo de investigación en un trabajo liderado por el Lic. Martín Hernández (Hernández *et al.*, 2015).

La investigación se basó en el análisis de dos regiones genómicas: la región hipervariable de VP2 y una porción del gen de la proteína VP1, el primero correspondiente al segmento A y el segundo al segmento B.

La región hipervariable del gen VP2 es la zona más variable de todo el genoma del virus y permite diferenciar de forma robusta las diferentes cepas circulantes a nivel mundial, debido a lo cual es la región más utilizada para la caracterización de nuevos brotes de IBDV. Los análisis filogenéticos de esta región separan a IBDV en cuatro clados integrados por: i) cepas clásicas (también llamadas clásicas virulentas), que incluye las primeras cepas reportadas de IBDV, así como cepas más actuales que comparten sus características genéticas, ii) cepas clásicas atenuadas o clásicas vacunales, compuesto por cepas clásicas que han sido modificadas en el laboratorio, como cepas vacunales o cepas adaptadas a crecimiento en diferentes cultivos celulares, iii) cepas variantes y iv) cepas hipervirulentas. El análisis filogenético de esta región incluyendo a las cepas *distinct* mostró claramente que éstas forman un linaje diferente, independiente de los linajes correspondientes al resto de las cepas de IBDV (Fig. 2 en Hernández *et al.*, 2015). En el análisis de los residuos aminoacídicos localizados en esta región, se encontraron algunos marcadores exclusivos de las cepas *distinct* (T272, P289, I290 y F296) (Fig. 1 en Hernández *et al.*, 2015). La presencia de cambios aminoacídicos en

la región hipervariable de VP2 es interesante desde el punto de vista antigénico, ya que al corresponder con la región más expuesta de la partícula viral suelen estar relacionados con alteraciones a nivel antigénico.

Con respecto al análisis del segmento B, es importante indicar que las relaciones filogenéticas normalmente obtenidas son diferentes a las observadas en el segmento A. En este último, las cepas del serotipo 2 ocupan una posición basal y se diferencian de los linajes de las demás cepas. En el análisis del segmento B, en cambio, las cepas hipervirulentas cambian su ubicación pasando a ser el linaje basal, mientras que las cepas clásicas, clásicas atenuadas y variantes se asocian en un único linaje. La hipótesis más aceptada actualmente para explicar el cambio observado en las cepas hipervirulentas propone que en el origen de este grupo viral ocurrió un evento de reordenamiento genómico, en donde el segmento A de un IBDV de serotipo 1 (posiblemente de cepa clásica) se combinó con el segmento B de un IBDV de tipo desconocido, no perteneciente al serotipo 1 ni 2 (Hon *et al.*, 2006). Por otro lado, el hecho de que las cepas clásicas, clásicas atenuadas y variantes no se diferencien evidencia una menor capacidad discriminatoria del segmento B. Sin embargo, como resultado del análisis de un fragmento del segmento B incluyendo cepas *distinct*, estas se diferenciaron del resto de las cepas formando un linaje aparte, estrechamente emparentado con el linaje de las cepas clásicas, clásicas atenuadas y variantes (Fig. 5 en Hernández *et al.*, 2015). Esto indica que las cepas *distinct* tienen un alto grado de divergencia con respecto al resto de las cepas, reforzando la idea de que se trataría de un grupo viral ancestral. En cuanto al análisis de las secuencias aminoacídicas, se vio la presencia de un marcador propio de este tipo de cepas (P243) en el fragmento analizado del segmento B (Fig. 4 en Hernández *et al.*, 2015).

Para analizar el grado de divergencia genética de las cepas de IBDV se realizó un estudio de DAPC (*Discriminant Analysis of Principal Components*). Este es un método multivariado para identificar y describir grupos de individuos genéticamente relacionados. Una de sus principales características es que permite maximizar la diferencia entre grupos, y por lo tanto su identificación, de una forma computacionalmente menos exigente que otros métodos similares. El análisis utilizando secuencias de la región hipervariable de VP2 evidenció el agrupamiento de los virus en cinco grupos, correspondientes a los linajes formados por las cepas clásicas, clásicas atenuadas, variantes, hipervirulentas y *distinct* obtenidos en el análisis filogenético de la misma región genómica (Fig. 3 en Hernández *et al.*, 2015). Además, permitió confirmar que las cepas *distinct* son de las más divergentes, junto con las cepas hipervirulentas.

Para completar la caracterización genética de este grupo viral, se obtuvo el primer genoma codificante completo de una cepa *distinct*, la cepa uruguaya dIBDV/UY/2014/2202 (Tomás *et al.*, 2015). El análisis de esta cepa no reveló cambios en cuanto al tamaño o localización de los genes virales. El análisis comparativo de ambos segmentos genómicos indicó una mayor similitud nucleotídica con cepas clásicas colectadas en los años 1960s, como la cepa Edgar (96% similitud, segmento A) y la cepa Irwin Moulthrop (96,4% similitud, segmento B).

En resumen, en este primer capítulo se evidencia que las cepas *distinct* son un grupo viral con características genéticas únicas. El genoma obtenido fue el primero para una cepa *distinct* y actualmente es considerado el genoma de referencia para estas cepas.

# CAPÍTULO 2- DESARROLLO DE UN MÉTODO DE DETECCIÓN ESPECÍFICO DE LAS CEPAS DISTINCT

En el capítulo anterior se describió la existencia de las cepas *distinct* como un grupo viral de circulación mundial con características genéticas únicas. Un rasgo interesante de estas cepas es el hecho de haber pasado prácticamente desapercibidas durante mucho tiempo, a pesar de haber circulado en varios países de América, Europa y Asia desde los años 1970s. Además de la aparente falta de signos clínicos provocados por estas cepas, tema que se retomará en el capítulo 4, otro factor que seguramente colaboró con el sub-diagnóstico de las cepas *distinct* es la falta de técnicas de detección específicas, como las existentes para las cepas variantes e hipervirulentas (Peters *et al.*, 2005; Kong *et al.*, 2009; Ghorashi *et al.*, 2011; Tomás *et al.*, 2012).

En el presente capítulo se describe el desarrollo de una metodología que detecta exclusivamente a las cepas *distinct*, diferenciándolas del resto de cepas de IBDV (Tomás *et al.*, 2017). Esta metodología está basada en la técnica de PCR en tiempo real, utilizando sondas de hidrólisis de alta especificidad (*minor groove binder* o MGB) para lograr una mayor especificidad. Los análisis genéticos realizados en el capítulo anterior revelaron la presencia de marcadores aminoacídicos específicos de las cepas *distinct*, tanto en la región hipervariable de VP2 como en una región parcial de VP1. La presente metodología utiliza los marcadores encontrados en VP2 para realizar el diseño de los cebadores y sondas. La utilización de marcadores de VP2 obedece a dos hechos: i) la región hipervariable de VP2 es la única secuenciada en la mayoría de las cepas de IBDV, incluidas las cepas *distinct*, y ii) los marcadores de esta región están muy conservados entre las cepas *distinct*, incluso entre cepas colectadas con más de 40 años de diferencia y en regiones geográficas muy alejadas.

La especificidad de la técnica comenzó en el diseño de cebadores. La combinación de cebador directo y reverso es 100% específica únicamente con las cepas *distinct*, destacándose un *mismatch* en la posición 3' del cebador reverse que está presente en todas las cepas no-*distinct*. La región en donde se diseñó la sonda MGB tiene entre dos y cuatro *mismatch* con cepas no-*distinct* (Fig. 1 en Tomás *et al*., 2017). Esto asegura una buena especificidad teniendo en cuenta que un único *mismatch* es suficiente para disminuir notoriamente la eficiencia de hibridación en las sondas MGB (Kutyavin *et al*., 2000). Dado que la región utilizada tiene algunas diferencias nucleotídicas entre las propias cepas *distinct*, fue necesario diseñar los cebadores y la sonda MGB con bases degeneradas.

La metodología desarrollada demostró una alta especificidad, no detectándose señal de amplificación frente a cepas no-*distinct* ni cuando se analizaron otros patógenos virales. Todas las cepas previamente clasificadas como *distinct* mediante secuenciación parcial de su genoma fueron correctamente clasificadas. En cuanto a la evaluación de parámetros analíticos del ensayo, se demostró que el mismo tiene un amplio rango dinámico ($10^3$-$10^8$ copias de ARN/reacción), un buen coeficiente de determinación ($R^2$= 0.9991) y un valor aceptable de eficiencia (Fig. 2 en Tomás *et al*., 2017). En conjunto, estos resultados demuestran que la metodología cumple con las condiciones necesarias para ser aplicada en la detección específica de las cepas *distinct*.

# CAPÍTULO 3- ESTUDIO FILODINÁMICO Y FILOGEOGRÁFICO DE LAS CEPAS DISTINCT

En este capítulo se realizan nuevos análisis filogenéticos con un mayor número de cepas *distinct*, y se analiza el origen y rutas de migración de estas cepas a través de abordajes filodinámicos. Los resultados presentados fueron aceptados con correcciones menores para su publicación en la revista *Transboundary and Emerging Diseases.*

Una de las características más llamativas de las cepas *distinct* es su antigüedad; las primeras cepas pertenecientes a este linaje fueron colectadas en Hungría en el año 1977. Para posicionar estas cepas en la historia evolutiva de IBDV, realizamos un análisis temporal comparativo y estimamos el año en el cual se habrían originado las cepas *distinct* en relación a las clásicas, variantes e hipervirulentas. Por otro lado, se realizó un estudio filogeográfico sobre las cepas *distinct* para reconstruir los principales eventos migratorios de estas cepas. También se obtuvieron los genomas completos de varias cepas *distinct* colectadas en Uruguay y Argentina, con las cuales se realizaron análisis filogenéticos y de marcadores aminoacídicos.

El estudio filogenético de los segmentos genómicos A y B reveló las mismas relaciones filogenéticas que habíamos obtenido en el análisis de fragmentos parciales, evidenciando que las cepas *distinct* son un linaje claramente separado de las demás cepas de IBDV (Fig. 1 & 2 en Tomás *et al*. 2020). El hecho de que el segmento B permita diferenciar a las cepas *distinct* como un linaje independiente de las cepas clásicas, clásicas atenuadas y variantes, puede explicarse al menos de dos maneras: i) las cepas *distinct* serían un grupo de virus ancestrales, y por lo tanto han divergido significativamente del resto de las cepas, y/o ii) las cepas *distinct* habrían evolucionado bajo tasas de sustitución más elevadas que el resto de las cepas. Los análisis de coalescencia realizados arrojan luz sobre este punto, lo cual es discutido en los próximos párrafos.

El análisis de marcadores aminoacídicos permitió ampliar el conocimiento preexistente sobre marcadores exclusivos de las cepas *distinct* (Tabla 2 en Tomás *et al*. 2020). En este sentido, se encontraron varios residuos marcadores de estas cepas. Se trata de marcadores potenciales porque el único genoma completo de una cepa *distinct* disponible en la base de datos era el correspondiente a la cepa uruguaya de referencia nombrada en el capítulo 1, y que por lo tanto algunas regiones genómicas están únicamente representadas por las cepas uruguayas y argentinas obtenidas en esta investigación (Fig. 3 en Tomás *et al*., 2020). Como se propone en el trabajo, sería necesario obtener el genoma completo de otras cepas *distinct*, idealmente representantes de la variabilidad genética y geográfica existente, para poder establecer cuáles serían realmente marcadores robustos. Al margen de lo dicho, se encontraron potenciales marcadores de cepas *distinct* en todas las proteínas del virus, cuyas implicancias biológicas se desconocen y deberían ser analizadas en futuros trabajos.

El análisis de coalescencia ubicó al ancestro común más reciente (*MRCA*) de las cepas clásicas y al de las cepas *distinct* muy cercanos en el tiempo, alrededor del año 1930 (Fig. 4 en Tomás *et al*., 2020). Por otro lado, el *MRCA* de las cepas variantes fue datado alrededor del año 1949, mientras que el de las cepas hipervirulentas se estableció alrededor del año 1967. Estos resultados sustentan fuertemente la hipótesis planteada sobre el origen ancestral de las cepas *distinct*. El año de origen estimado para las cepas hipervirulentas (1967) es muy similar al estimado por Hon *et al.* (2006) y Alfonso-Morales *et al.* (2013) ubicado en los años 1962 y 1970 respectivamente. El año de origen de las cepas variantes (1949) fue estimado por primera vez en esta Tesis.

En cuanto a los eventos migratorios estimados para las cepas *distinct*, se destacan tres migraciones intercontinentales ocurridas desde Europa del Este, lugar más probable para el origen de estas cepas, hacia Asia del Este (1959), Brasil (1963) y Argentina (1990) (Fig. 5 en Tomás *et al*., 2020). Considerando la llegada de las cepas *distinct* a Uruguay, este evento habría ocurrido recientemente (2005-2006) desde Argentina.

Es difícil establecer vínculos directos entre los eventos de migración con hechos puntuales que los hayan favorecido, sin embargo, existen algunos datos que se alinean con esas estimaciones. Por ejemplo, el hecho de que Hungría, uno de los dos países con mayor probabilidad de ser origen de las cepas *distinct*, haya incrementado notoriamente las exportaciones de productos avícolas a partir de los años 1960s puede interpretarse como un hecho que favoreció la diseminación de las cepas *distinct* desde Europa hacia otros continentes. Por otro lado, también hay que considerar la posibilidad de que el virus se haya diseminado hacia países cercanos y que a partir de estos haya alcanzado otros continentes; a pesar de que no existan registros de cepas *distinct* en países que eran de los principales exportadores de pollos vivos y productos avícolas en la década de los 1960s, como Holanda, Francia y Bélgica, esta teoría no debería ser descartada.

Haciendo un análisis de las relaciones filogenéticas obtenidas dentro del linaje de las cepas *distinct*, es notoria la correlación encontrada con los patrones de migración estimados, pudiendo subdividir a este linaje en tres clados: i) clado formado por cepas *distinct* brasileñas, las cuales se habrían originado a partir de un único evento migratorio y que comparte marcadores aminoacídicos no presentes en las otras cepas *distinct*, y que incluye también una cepa de Emiratos Árabes Unidos con los mismos marcadores aminoacídicos, ii) clado formado por cepas asiáticas (Corea del Sur y Japón) y

canadienses, donde las segundas se habrían originado a partir de un evento migratorio desde las primeras, y que también incluye dos cepas de Arabia Saudita que habrían llegado a ese país recientemente desde Canadá, y iii) clado formado por cepas polacas, húngaras, argentinas y uruguayas, las cuales comparten varios marcadores aminoacídicos que definen a este clado.

Los resultados obtenidos en este capítulo arrojan luz sobre la evolución de IBDV y demuestran que las cepas *distinct* habrían sido contemporáneas de las clásicas desde etapas tempranas de su historia, y que diferentes eventos de migración intercontinentales son la explicación de la variabilidad y distribución de estos virus que detectamos en la actualidad.

# CAPÍTULO 4- ESTUDIO DE LA ANTIGENICIDAD, PATOGENICIDAD Y CAPACIDAD INMUNODEPRESORA DE LAS CEPAS DISTINCT

Los estudios presentados en este capítulo se realizaron en el marco de una colaboración con el laboratorio del Dr. Nicolas Eterradossi, el cual es referencia para la OIE (Organización Internacional de Sanidad Animal) en el estudio mundial de IBDV. A través de una pasantía de dos meses de duración que realicé en dicho laboratorio pude efectuar esta investigación utilizando las mismas instalaciones, aves y protocolos empleados para estudiar otras cepas de IBDV, lo cual reduce variables y por lo tanto favorece la posibilidad de comparar los resultados. Este es uno de los puntos más complejos y críticos de este tipo de estudios donde se realizan infecciones experimentales en aves.

El estudio de antigenicidad se basó en la utilización de un panel de ocho anticuerpos monoclonales, ampliamente utilizados para la caracterización antigénica de cepas de IBDV (Eterradossi *et al.*, 2004; Martin *et al.*, 2007; Li *et al.*, 2015; Abed *et al.*, 2018; Samy *et al.*, 2020). El estudio consiste en medir la afinidad de un determinado aislamiento viral con cada uno de los anticuerpos en un ensayo de ELISA de captura antigénica. Los resultados indicaron, por un lado, que la cepa *distinct* uruguaya mantiene un perfil antigénico muy similar al de las cepas *distinct* polacas aisladas hace más de 30 años, y por otro que ese perfil es notoriamente diferente al del resto de las cepas tradicionales de IBDV. Estos hallazgos indican que las cepas *distinct* son antigénicamente diferentes, lo cual podría repercutir en una disminución de la protección vacunal generada con las vacunas comerciales desarrolladas hasta ahora, ninguna de las cuales incluye una cepa *distinct* en su formulación. Sin embargo, esta problemática debería ser evaluada de forma directa a través de ensayos de protección vacunal para poder establecer efectivamente si existe una adecuada protección cruzada.

El ensayo de patogenicidad implicó la infección de un grupo de aves SPF con una cantidad conocida de virus de cepa *distinct*, además de contar con un grupo control infectado con una cepa hipervirulenta y un grupo control sin infectar. A lo largo del ensayo se registraron los signos clínicos observados, se pesaron las aves previo a la realización de necropsias a los 4 y 21 días post infección, y se determinaron las lesiones en diferentes órganos y el peso de la bursa de Fabricio; posteriormente se tomaron muestras de sangre. La cepa *distinct* no provocó ningún signo clínico en las aves infectadas, lo cual concuerda con las observaciones previas (Fig. 2A & B en Tomás *et al*., 2019). En cuanto a los daños observados en las necropsias, la única alteración detectada en forma consistente fue la atrofia de la bursa de Fabricio, particularmente en el día 21 pi en comparación con el día 4 pi (Fig. 2C en Tomás *et al*., 2019). Por lo tanto, concluimos que la cepa *distinct* provoca daños notorios en la bursa de Fabricio sin generar signos clínicos evidentes.

Luego de observar estos resultados se evaluó si el nivel de daño detectado en la bursa de Fabricio se traducía en una inmunodepresión de las aves. La forma en la que se midió la inmunodepresión fue la misma utilizada previamente para otras cepas de IBDV, en donde se evalúa en nivel de protección generado por una vacuna del virus de Newcastle (NDV) al desafío con una cepa de NDV virulenta, en aves que previamente fueron infectadas con la cepa de IBDV. La lógica del ensayo implica que, si la cepa de IBDV provoca inmunodepresión, la vacuna de NDV aplicada posteriormente no generará una protección adecuada y por lo tanto, se observará mortalidad cuando las aves sean desafiadas con la cepa de NDV virulenta. Además de un grupo de aves infectadas con IBDV, se utilizaron dos grupos de aves control sin infectar. El primero es el grupo control vacunado, al cual se le aplicó la vacuna de NDV y posteriormente el desafío con la cepa de NDV virulenta, y el segundo es el control sin vacunar, al cual sólo se lo desafió. Se tomaron

muestras de sangre de aves de todos los grupos previo al desafío para medir el nivel de anticuerpos generados por la vacuna de NDV. Además de la cepa *distinct* uruguaya, también se realizó en paralelo el análisis de la cepa *distinct* polaca. Los resultados indicaron que las aves infectadas con ambas cepas *distinct* provocaron inmunodepresión, evidenciado por la notoria disminución en el nivel de anticuerpos generados en respuesta a la vacuna de NDV, y a la aparición de niveles de mortalidad elevados tras el desafío con la cepa de NDV virulenta (Fig. 3 en Tomás *et al.*, 2019).

Los resultados de todo este trabajo concluyen que las cepas *distinct* son un grupo de virus antigénicamente diferentes a las cepas tradicionales de IBDV, y que son capaces de infectar a las aves provocando daños principalmente en la bursa de Fabricio.

# CONSIDERACIONES GENERALES

La avicultura es una industria que ha mostrado un crecimiento constante en los últimos años, tendencia que se mantendrá en el futuro según proyecciones recientes (Fig. 9). Una de las características que favorece el crecimiento de esta industria es la crianza de tipo intensivo que se practica, gracias a lo cual se pueden generar altísimos niveles de producción ocupando mínimos espacios físicos. Sin embargo, este tipo de crianza inevitablemente promueve la aparición y diseminación de enfermedades infecciosas, por lo cual es un tema que debe ser muy bien atendido para obtener niveles adecuados de producción. Es en ese sentido que los estudios realizados en esta tesis apuntaron a ampliar el conocimiento acerca del IBDV, uno de los patógenos más frecuentemente observados en la avicultura a nivel mundial.

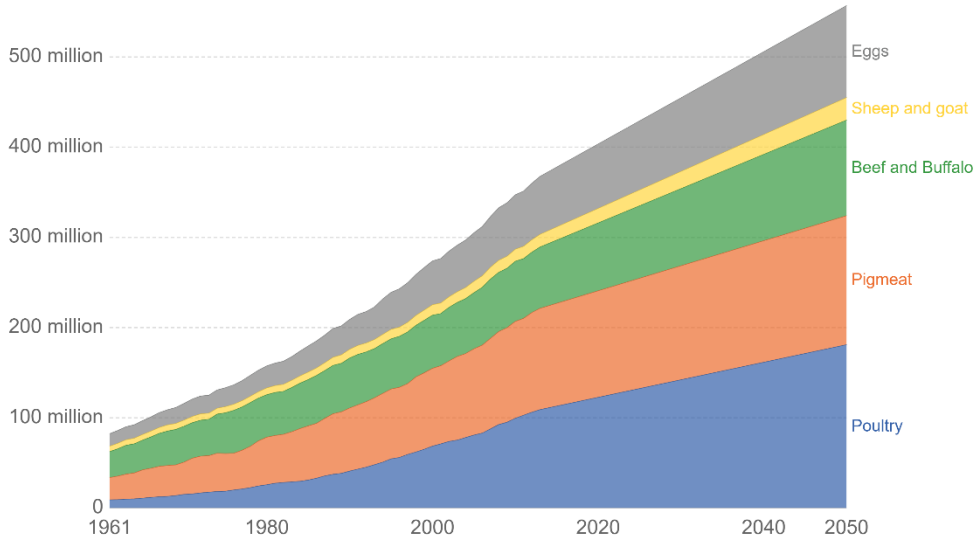

Figura 9- Proyecciones al 2050 de la producción mundial de huevos y carne por especie. Gráfico tomado y modificado de http://www.ourworldindata.org, según datos obtenidos de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO).

Nuestros estudios se centraron inicialmente en la caracterización genética y el estudio de la evolución de este virus, para luego analizar aspectos relacionados con la biología del virus y las consecuencias de la interacción con su hospedero.

Describiendo de forma global lo que ha sido la historia evolutiva del IBDV, se puede considerar que existen tres hitos principales: i) la primera descripción de la enfermedad a principios de los años 1960s, asociada a las cepas conocidas hoy como clásicas o clásicas virulentas; ii) el surgimiento en EEUU a mediados de los años 1980s de cepas con características antigénicas diferentes a las de las cepas clásicas, conocidas como cepas variantes; y iii) la aparición en Europa a fines de los años 1980s de cepas que provocaban altas tasas de mortalidad en ausencia de grandes cambios a nivel antigénico, llamadas hoy cepas hipervirulentas o muy virulentas (Cosgrove, 1962; Rosenberger *et al.*, 1985; van den Berg *et al.*, 1991). Esto no significa que a lo largo de la historia del virus no existan reportes de otras cepas con características patogénicas, antigénicas y/o genéticas diferentes a las recién nombradas, como por ejemplo los casos de las cepas que circulan en Australia o en Italia (Ignjatovic & Sapats, 2002; Lupini *et al.*, 2016). Sin embargo, las cepas australianas e italianas se encuentren circulando en regiones geográficas limitadas, lo que las posiciona en un segundo plano en cuanto a su impacto epidemiológico y evolutivo.

En la primera parte de esta Tesis se describe la existencia de un nuevo tipo de cepa de IBDV con características genéticas propias y distintivas, por lo que fueron denominadas *distinct*. Una de las principales características de estas cepas es que se han encontrado en varios países de tres continentes (Norte y Sudamérica, Europa y Asia). Este carácter global, que solo se había observado en las cepas clásicas, variantes e hipervirulentas, coloca a las cepas *distinct* en una posición equivalente a la de las cepas tradicionales en cuanto a su importancia epidemiológica y evolutiva.

A través de análisis filogenéticos basados en fragmentos de ambos segmentos genómicos, así como también al análisis de marcadores aminoacídicos y al análisis de DAPC, se demostró la existencia de este grupo viral y se describieron las principales características genéticas del mismo, obteniendo incluso el primer genoma completo de una cepa *distinct* (Fig. 10) (Hernández *et al.*, 2015; Tomás *et al.*, 2015).

Una característica particular de este trabajo es que no trata sobre la descripción de una cepa nueva de IBDV, en el sentido de que ya existían diversos trabajos que reportaban la presencia de estas cepas, muchos de los cuales generaron secuencias que están disponibles en la base de datos para su libre utilización y análisis desde hace años. Sin embargo, cada uno de esos reportes describía a estas cepas como variantes locales raras, sin hacer una vinculación con los otros reportes ya existentes. Esto provocó que no existiera una conciencia a nivel global de la existencia de estas cepas. El principal valor y aporte de este trabajo es el de haber estudiado de forma exhaustiva la base de datos de secuencias y la bibliografía, vinculando las cepas más frecuentemente encontradas en Uruguay con reportes previos de esa misma cepa en otras partes del mundo. Recién en el momento de unificar toda esa información que se encontraba fragmentada pudimos visualizar que lo que

circulaba en nuestro país era algo más que una simple diferenciación local, y así lo presentamos en el trabajo de Hernández et al. (2015).

Es importante mencionar que poco tiempo después se conoció una publicación que propone una clasificación actualizada de las cepas de IBDV basado en análisis filogenéticos la cual incluye a las cepas *distinct*, y que trabajos más recientes acerca de la caracterización de cepas de IBDV en distintos países han incorporado esa propuesta incluyendo cepas *distinct* en sus análisis (Michel & Jackwood, 2017; Jackwood *et al.*, 2018; Molini *et al.*, 2019; Yilmaz *et al.*, 2019; Samy *et al.*, 2020)

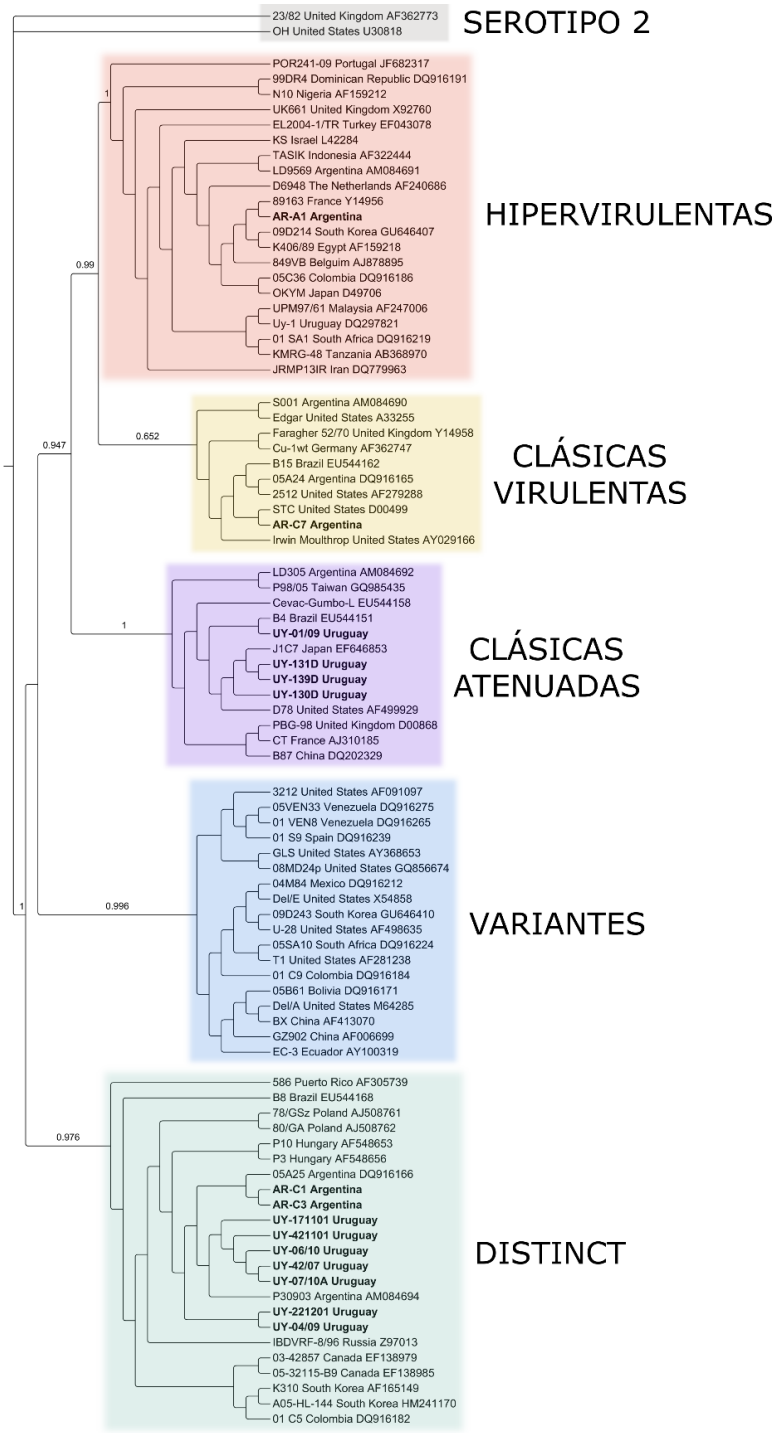


Figura 10- Árbol filogenético de IBDV realizado en base a la región hipervariable de VP2. Las cepas *distinct* descritas en esta Tesis se localizan juntas en un clado independiente con un alto soporte estadístico. Tomado y modificado de Hernández *et al.* (2015).

Luego de haber descrito por primera vez la existencia de las cepas *distinct* como un grupo viral de circulación mundial, y por lo tanto de relevancia epidemiológica, nos propusimos el desarrollo de una metodología rápida, sensible y específica para facilitar su detección. Para esto, nos dedicamos al desarrollo de una herramienta basada en PCR en tiempo real capaz de detectar específicamente las cepas *distinct* (Fig. 11) (Tomás *et al.*, 2017).

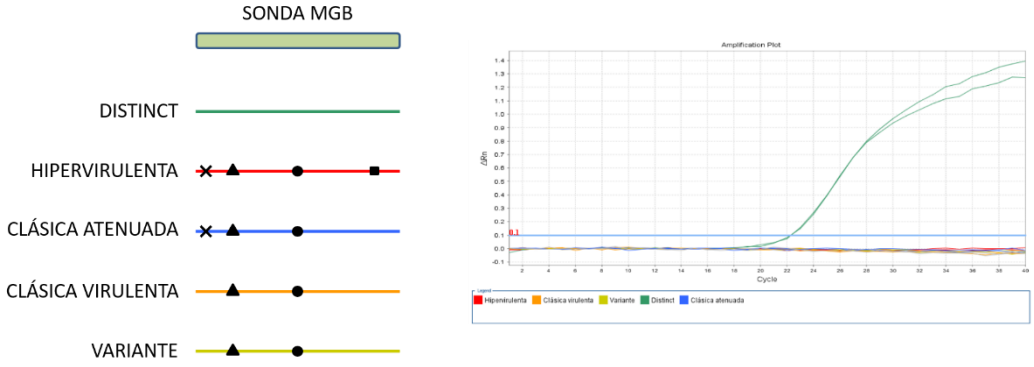


Figura 11- Ensayo de PCR en tiempo real para la detección específica de las cepas *distinct.* Esquema representando la homología de la sonda MGB con cada una de las cepas, donde cada nucleótido de diferencia se representa con un símbolo diferente de color negro (izquierda). Gráfico de amplificación correspondiente a la evaluación de la especificidad del ensayo, donde se observa amplificación solo en el caso de la muestra *distinct* y no en el resto de las cepas (derecha).

El diseño de la técnica fue un desafío importante ya que hubo que trabajar sobre una de las regiones más variables del genoma del virus, debido a que prácticamente era la única región con secuencias de cepas *distinct* disponibles. Sin embargo, gracias a la presencia de marcadores exclusivos y muy conservados dentro de estas cepas, sumado al uso de una sonda de tipo MGB, fue posible hacer un ensayo que funciona de forma adecuada. Esto

quedó demostrado mediante la realización de varias pruebas durante la validación del método.

Así se obtuvo una herramienta sensible y robusta para aplicar en estudios de diagnóstico y caracterización de IBDV, favoreciendo la identificación de estas cepas que históricamente se habían mantenido circulando de forma silenciosa. Esta metodología permite diagnosticar y caracterizar simultáneamente el virus en forma rápida y certera, por lo cual está siendo utilizada en forma rutinaria en nuestro laboratorio. Una utilidad particular de este ensayo es la posibilidad de emplearse para estudios que impliquen la coinfección de distintas cepas en las mismas aves o cultivos, e incluso su cuantificación, ya que se puede hacer la detección y seguimiento de la cepa *distinct* de forma independiente. Esto es particularmente útil por ejemplo en estudios de protección vacunal, en donde eventualmente la cepa vacunal evaluada y la cepa *distinct* utilizada como desafío coexistirían en un mismo animal.

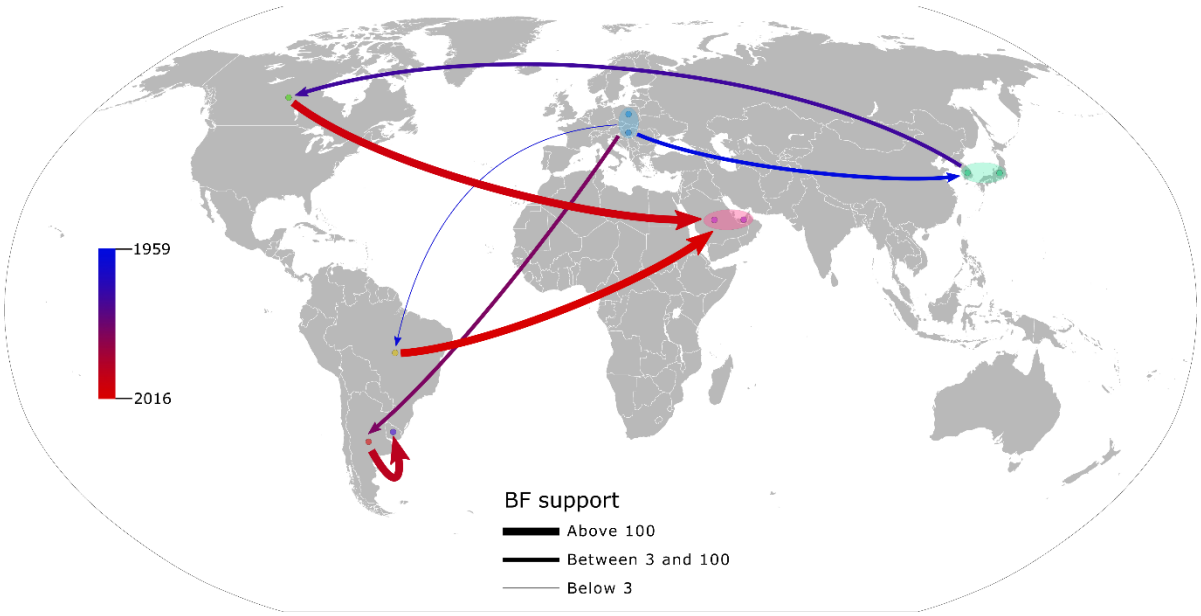

Figura 12- Esquema representando los principales eventos migratorios obtenidos en el análisis filogeográfico de las cepas *distinct*. Con tonalidades de colores que van del azul al rojo se indican los eventos ocurridos hace más y menos años respectivamente. El grosor de las flechas representa el soporte estadístico calculado para cada evento mediante el valor de Bayes Factor (BF). Tomado de Tomás *et al.* (2020).

Posteriormente nos centramos en el estudio temporal de las cepas *distinct* desde el punto de vista evolutivo, estimando su año de origen y comparándolo con el del resto de cepas tradicionales de IBDV, así como también determinando el lugar geográfico más probable de su origen y su posterior migración a otras regiones (Fig. 12) (Tomás *et al.*, 2020).

Nuestros análisis indican que las cepas distinct son antiguas y que se habrían originado aproximadamente al mismo tiempo que las cepas clásicas (Fig. 13). Esta hipótesis del origen de las cepas *distinct* es importante porque cambia el paradigma de evolución para este virus, introduciendo un nuevo protagonista que habría participado de forma temprana en la evolución del IBDV. El hecho de que las cepas *distinct* sean tan antiguas y hayan alcanzado una distribución tan extendida sustentan su importancia en la historia evolutiva del virus. Casi dos y cuatro décadas después del origen de las cepas clásicas y *distinct* se habrían originado las cepas variantes e hipervirulentas, respectivamente.

Un punto interesante es el notorio desfasaje que hay entre los años estimados de origen de cada cepa y los años en que fueron reportadas por primera vez (Fig. 13).

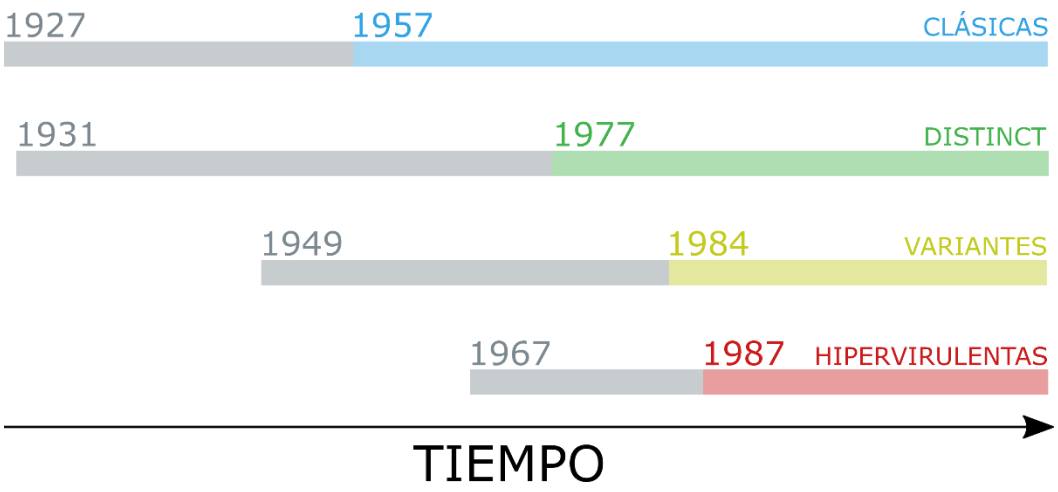


Figura 13- Representación esquemática de los años de origen (gris) y los años en los cuales fueron reportados por primera vez (colores) las cepas clásicas, variantes, hipervirulentas y *distinct*.

Esto puede explicarse por diversas causas, entre las que se destacan: i) la capacidad de provocar signos clínicos detectables por veterinarios y/o productores avícolas pudo haber surgido posteriormente al surgimiento del ancestro común de cada cepa, el cual fue acumulando distintos tipo de mutaciones provocando cambios en la interacción virus-hospedero, ii) la propia historia de la industria avícola fue cambiando a lo largo de los años, modificando aspectos como la nutrición, las condiciones intensivas de cría, las líneas genéticas de las aves utilizadas para carne y huevo, el comercio de los productos avícolas, etc., generando condiciones ecológicas diferentes, posiblemente más adecuadas para el surgimiento de enfermedades virales, y iii) el desarrollo y aplicación de vacunas para controlar los brotes de IBDV, luego de la aparición de las cepas clásicas, posiblemente ejerció una fuerza evolutiva importante que actuó no solo sobre las propias cepas clásicas sino también sobre los ancestros de las cepas variantes e hipervirulentas actuales que ya se encontraban circulando.

A modo de conclusión del análisis temporal y geográfico de las cepas *distinct*, además de generar nuevas secuencias genómicas, se puede decir que se sumaron evidencias a favor de la hipótesis que proponía a las cepas *distinct* como un grupo viral ancestral dentro de la historia de IBDV. Esto es muy interesante desde el punto de vista epidemiológico y evolutivo, ya que, a diferencia del linaje de las cepas clásicas que originó las cepas variantes e hipervirulentas, las cepas *distinct* nunca evolucionaron hacia un estado muy patogénico que generara problemas sanitarios notorios. Por el contrario, su descripción como un grupo viral epidemiológicamente relevante fue hecho como consecuencia de la aplicación de abordajes genéticos, los cuales han ganado relevancia en los últimos años favorecidos por la facilidad creciente de generar y acceder a las secuencias genómicas de los virus (Wu *et al.*, 2007).

En conjunto, nuestros hallazgos indican que la evolución de IBDV ha sido más compleja de lo que hace unos años se pensaba, y que es necesario continuar con estudios de este tipo incorporando más datos de secuencias para poder aclarar aún más la dinámica evolutiva de IBDV e intentar aplicar este conocimiento al desarrollo de estrategias de control específicas y más eficientes. Creemos que estos resultados sustentan el papel de IBDV como un virus que puede utilizarse como modelo evolutivo para estudiar el origen y dispersión de un patógeno estrechamente asociado a la cría intensiva de aves.

En la última parte de esta Tesis nos realizamos interrogantes acerca de las características biológicas de las cepas *distinct.* Trabajos previos con cepas hoy identificadas como *distinct,* indicaban que las aves infectadas no mostraban signos clínicos de IBDV, y que incluso en algunos casos el aislamiento fue realizado de aves aparentemente sanas. Por otro lado, trabajos que

involucraron infecciones controladas realizados hace más de 30 años, sugerían que cepas distinct europeas no tenían signos clínicos o estos eran muy leves (Minta *et al.*, 1985a). Sin embargo, presentaban un notorio daño de la bursa de Fabricio e inmunodepresión (Minta *et al.*, 1985b). Estas mismas cepas *distinct* europeas presentaban cambios antigénicos con respecto a cepas de IBDV tradicionales (Domanska *et al.*, 2004). Teniendo en cuenta estos antecedentes, nos propusimos estudiar las cepas *distinct* que circulan actualmente en Uruguay, evaluando su patogenicidad, antigenicidad y capacidad inmunodepresora en comparación con cepas tradicionales de referencia y las cepas *distinct* antiguas anteriormente estudiadas (Tomás *et al.*, 2019).

Los hallazgos de estos estudios indicaron que la cepa *distinct* uruguaya presenta notorios cambios antigénicos con respecto a las cepas tradicionales y que se mantiene muy similar a la cepa *distinct* europea analizada años atrás (Fig. 14). Estos cambios antigénicos deberían ser analizados en mayor profundidad por su potencial implicancia en el control del virus. Es inevitable con estos resultados cuestionarse la eficacia que tendrán las vacunas que se aplican en nuestro país para controlar a la cepa *distinct.* Estudios de neutralización cruzada, o mejor aún estudios de protección vacunal, serían necesarios para poder tener una respuesta más acertada acerca de ese tema.

| Cepa | Mab 1 | Mab 3 | Mab 4 | Mab 5 | Mab 6 | Mab 7 | Mab 8 | Mab 9 |
|---|---|---|---|---|---|---|---|---|
| F52/70 | 59 | 58 | 70 | 48 | 100 | 73 | 109 | 78 |
| 89163 | 58 | 1 | 6 | 27 | 95 | 86 | 76 | 73 |
| Variante E | - | 2 | - | - | 6 | - | 8 | - |
| *Distinct* polaca | 32 | -1 | 0 | -1 | 101 | 96 | 23 | 91 |
| *Distinct* uruguaya | 26 | -1 | 0 | 0 | 105 | 90 | 26 | 73 |

Figura 14- Afinidad de 8 anticuerpos monoclonales frente a una cepa clásica (F52/70), hipervirulenta (89163), variante (Variante E), *distinct* polaca y *distinct* uruguaya. El sombreado en gris claro representa afinidad media, y el sombreado en gris oscuro representa afinidad baja. El símbolo "-" indica que no se analizó ese anticuerpo. Valores tomados de Domanska *et al.* (2004) y Tomás *et al.* (2019).

Por otro lado, se determinó que la cepa *distinct* no provoca signos clínicos en las aves infectadas, pero afecta notoriamente la bursa de Fabricio (Fig. 15). Además, genera un grave estado de inmunodepresión al igual que las cepas *distinct* más antiguas. El hecho de que las cepas *distinct* generen niveles altos de inmunodepresión en ausencia de signos clínicos en las aves afectadas tiene una aplicación directa para el control de la enfermedad. Se trata de características sumamente problemáticas para la avicultura porque los virus pueden pasar inadvertidos, aunque afecten constantemente el sistema inmune de las aves. Esto favorece la aparición recurrente de diversas patologías, como las respiratorias o entéricas, y genera pérdidas constantes en la producción. Este conocimiento resulta importante para el veterinario ya que debe considerar la existencia de estas cepas en la alteración de los niveles de producción.

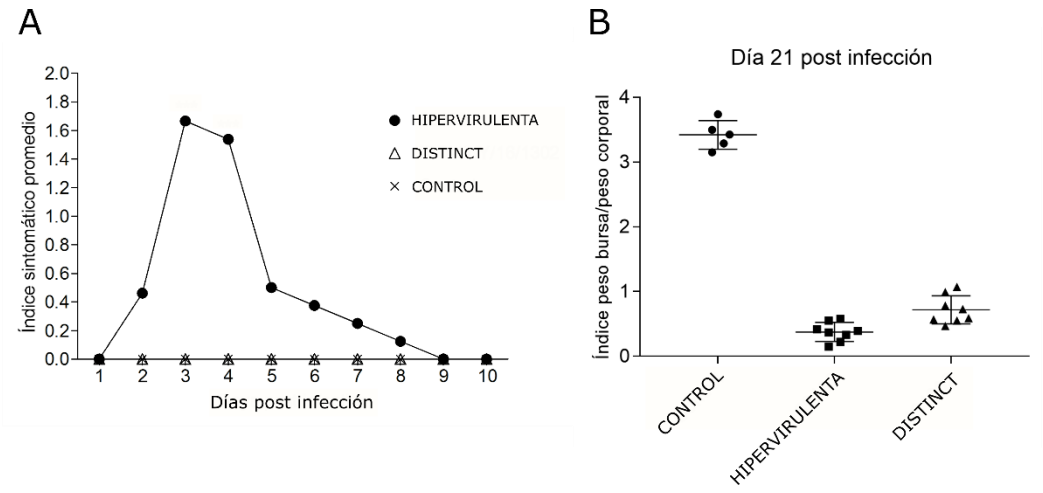


Figura 15- Signos clínicos observados tras la infección de aves SPF de 6 semanas de edad (A). Nivel de atrofia de la bursa de Fabricio 21 días post infección (B). Tomado y modificado de Tomás *et al.* (2019).

En conjunto, estos estudios permitieron avanzar en el conocimiento de las cepas *distinct* que circulan en la actualidad, particularmente en Uruguay. Esto adquiere mayor relevancia considerando que los estudios previos habían sido realizados en cepas *distinct* de otro continente y de hace más de tres décadas, lo que podría haber causado diferencias importantes con los virus que circulan actualmente en Sudamérica. Sin embargo, no se observaron grandes diferencias, evidenciando una sorprendente conservación de las características fenotípicas entre ambas cepas.

Al inicio de este trabajo pretendíamos caracterizar las cepas de IBDV circulantes en Uruguay, esperando encontrar principalmente cepas clásicas o quizás hipervirulentas, teniendo en cuenta los antecedentes en nuestro país y los reportes de países de la región existentes en ese momento (Di Fabio *et al.*, 1999; Gomes *et al.*, 2005; Hernández *et al.*, 2006). Sorpresivamente, nos encontramos con variantes atípicas, de las cuales había registros esporádicos en otras partes del mundo. Estas descripciones hacían referencia a estas cepas como variantes locales de circulación limitada a determinada región geográfica y período de tiempo. Nuestros estudios comparativos establecieron por primera vez que se trataba de un linaje antiguo y de expansión global, resultados que fueron sustentados por trabajos realizados en Argentina y Brasil (Vera *et al.*, 2015; de Fraga *et al.*, 2019). En ese momento este trabajo adquirió una nueva dimensión, y pasó de ser la descripción genética de una población local a identificar un linaje que aportaba nueva información a la historia evolutiva del IBDV. Pocas veces, tenemos la posibilidad desde Sudamérica de hacer un aporte relevante a la evolución de un virus de tanta importancia productiva. Gracias a esta investigación, junto con los trabajos realizados en el virus de la Bronquitis infecciosa aviar, pudimos posicionar a nuestro grupo como referente a nivel regional para el estudio de ambos patógenos.

# CONCLUSIONES

- Se caracterizó genéticamente un linaje viral previamente no definido (linaje *distinct*) y se demostró genéticamente que se trata de un grupo viral diferente al resto de las cepas tradicionales.
- Este linaje circula con alta frecuencia en las granjas avícolas uruguayas además de otros países de varios continentes.
- Se determinó el lugar y año estimado de su origen, así como también las rutas migratorias principales que generaron la distribución actual de este grupo viral, a través de lo cual se propuso un nuevo escenario evolutivo de IBDV y se aportó al conocimiento de la epidemiología a nivel regional y mundial.
- Se diseñó y validó un método rápido y robusto para el diagnóstico específico de las cepas *distinct* basado en la PCR en tiempo real y el uso de sondas de hidrólisis de tipo MGB.
- Se determinó la patogenicidad de una cepa *distinct* uruguaya a través de estudios en aves SPF, demostrando que se trata de una cepa que no provoca signos clínicos, pero sí una severa atrofia de la bursa de Fabricio asociada a una fuerte inmunodepresión.
- Se estudiaron las características antigénicas de una cepa *distinct* uruguaya mediante el uso de un panel de anticuerpos monoclonales, lo cual reveló la existencia de alteraciones antigénicas en comparación con el resto de las cepas tradicionales de IBDV.

# PERSPECTIVAS

Teniendo en cuenta que las cepas *distinct* continúan siendo el tipo de cepa más frecuente en Uruguay y la región (Argentina y Brasil), el trabajo a futuro pretende seguir en la misma línea a través de los siguientes estudios:

- Obtención de los genomas completos de cepas *distinct* de diferentes partes del mundo. Esto permitiría complementar el trabajo de esta Tesis y obtener un mejor conocimiento de la genética de estos virus y de los cambios que ha experimentado durante su evolución. En este sentido, pretendemos secuenciar cepas *distinct* de origen polaco colectadas en el período 1977-1980, a través de una colaboración con el laboratorio del Dr. Nicolas Eterradossi, Ploufragan, Francia. Adicionalmente, se intentará acceder a otras muestras, como por ejemplo de Corea del Sur, Japón, Canadá y Brasil, para obtener una mayor representación de los sublinajes existentes dentro de las cepas *distinct*.

- Comenzar a trabajar en el aislamiento de cepas *distinct* de campo, tanto uruguayas como argentinas, en huevos embrionados SPF y diferentes cultivos celulares. Este trabajo ya se inició en el marco de proyectos conjuntos con el laboratorio del Dr. Ariel Vagnozzi, INTA Castelar, Buenos Aires, y próximamente se pretende realizar en el país mediante el acondicionamiento de un laboratorio para trabajar con cultivos celulares y huevos embrionados SPF.

- Puesta a punto de protocolos para la realización de ensayos de neutralización viral, tanto en huevos embrionados SPF como en

cultivos celulares. Esto permitirá disponer de una herramienta para realizar análisis antigénicos sobre cepas *distinct* y cualquier otro tipo de cepa de IBDV. Este tipo de ensayo suele utilizarse como una alternativa más económica y menos demandante en cuanto a infraestructura para analizar el nivel de protección cruzada entre virus de diferentes cepas de IBDV.

- Realización de un estudio de protección vacunal en aves SPF, evaluando los niveles de protección generados por diferentes vacunas normalmente utilizadas en Uruguay y Argentina frente al desafío con una cepa *distinct*. Este trabajo es sumamente relevante desde un punto de vista productivo y pretendemos que sirva para mejorar el control de este patógeno en la región. Cabe mencionar que este estudio ya se encuentra en marcha en el marco de un proyecto María Viñas, en el cual participa el laboratorio del Dr. Ariel Vagnozzi y forma parte del trabajo de Doctorado de la Mag. Claudia Techera.

# REFERENCIAS


Abed M, Soubies S, Courtillon C, Briand FX, Allée C, Amelot M, Boisseson C De, Lucas P, Blanchard Y, Belahouel A, Kara R, Essalhi A, Temim S, Khelef D, Eterradossi N (2018) Infectious bursal disease virus in Algeria: Detection of highly pathogenic reassortant viruses. *Infection, Genetics and Evolution* **60**, 48–57.

Alfonso-Morales A, Martínez-Pérez O, Dolz R, Valle R, Perera CL, Bertran K, Frías MT, Majó N, Ganges L, Pérez LJ (2013) Spatiotemporal Phylogenetic Analysis and Molecular Characterisation of Infectious Bursal Disease Viruses Based on the VP2 Hyper-Variable Region. *PloS one* **8**, e65999.

Alfonso-Morales A, Rios L, Martínez-Pérez O, Dolz R, Valle R, Perera CL, Bertran K, Frías MT, Ganges L, Díaz de Arce H, Majó N, Núñez JI, Pérez LJ (2015) Evaluation of a Phylogenetic Marker Based on Genomic Segment B of Infectious Bursal Disease Virus: Facilitating a Feasible Incorporation of this Segment to the Molecular Epidemiology Studies for this Viral Agent. *PloS one* **10**, e0125853.

Allan WH, Faragher JT, Cullen GA (1972) Immunosuppression by the infectious bursal agent in chickens immunised against Newcastle disease. *The Veterinary record* **90**, 511–2.

Bayliss CD, Spies U, Shaw K, Peters RW, Papageorgiou A, Muller H, Boursnell MEG (1990) A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *Journal of General Virology* **71**, 1303–1312.

Benton WJ, Cover MS, Rosenberger JK, Lake RS (1967) Physicochemical Properties of the Infectious Bursal Agent (IBA). *Avian Diseases* **11**, 438.

Berg TP Van Den, Eterradossi N, Toquin D, Meulemans G (2000) Infectious bursal disease (Gumboro disease). *Revue Scientifique et Technique International Office of Epizootics* **19**, 509–543.

Berg TP van den, Gonze M, Meulemans G (1991) Acute infectious bursal disease in poultry: Isolation and characterisation of a highly virulent strain. *Avian pathology,* **20**, 133–43.

Birghan C (2000) A non-canonical Lon proteinase lacking the ATPase domain employs the Ser-Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus. *The EMBO Journal* **19**, 114–123.

Böttcher B, Kiselev NA, Stel'Mashchuk VY, Perevozchikova NA, Borisov A V, Crowther RA (1997) Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. *Journal of virology* **71**, 325–30.

Brandt M, Yao K, Liu M, Heckert RA, Vakharia VN (2001) Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. *Journal of virology* **75**, 11974–82.

Bublot M, Pritchard N, Gros FX Le, Goutebroze S (2007) Use of a Vectored Vaccine against Infectious Bursal Disease of Chickens in the Face of High-Titred Maternally Derived Antibody. *Journal of Comparative Pathology* **137**.

Bumstead N, Reece RL, Cook JK (1993) Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. *Poultry science* **72**, 403–410.

Castón JR, Martínez-Torrecuadrada JL, Maraver A, Lombardo E, Rodríguez JF, Casal JI, Carrascosa JL (2001) C terminus of infectious bursal disease virus major capsid protein VP2 is involved in definition of the T number for capsid assembly. *Journal of virology* **75**, 10815–28.

Chen F, Liu J, Yan Z, Liu D, Ji J, Qin J, Li H, Ma J, Bi Y, Xie Q (2012) Complete

genome sequence analysis of a natural reassortant infectious bursal disease virus in China. *Journal of virology* **86**, 11942–3.

Chettle N, Stuart JC, Wyeth PJ (1989) Outbreak of virulent infectious bursal disease in East Anglia. *The Veterinary record* **125**, 271–2.

Chettle NJ, Eddy RK, Wyeth PJ (1985) Comparison of virus neutralizing and precipitating antibodies to infectious bursal disease virus and their effect on susceptibility to challenge. *British Veterinary Journal* **141**, 146–150.

Chettle NJ, Wyeth PJ (1989) Failure of maternally derived infectious bursal disease antibodies to serotypes 1 and 2 to protect against heterologous virus. *British Veterinary Journal* **145**, 165–169.

Chevalier C, Galloux M, Pous J, Henry C, Denis J, Costa B Da, Navaza J, Lepault J, Delmas B (2005) Structural Peptides of a Nonenveloped Virus Are Involved in Assembly and Membrane Translocation. *Journal of Virology* **79**, 12253–12263.

Chin R, Yamamoto R, Lin W, Lam K, Farver T (1984) Serological survey of infectious bursal disease virus serotypes 1 and 2 in California turkeys. - PubMed - NCBI. *Avian Diseases* **28**, 1026–36.

Cho Y, Edgar SA (1969) Characterization of the infectious bursal agent. *Poultry science* **48**, 2102–2109.

Ciriaco E, Píñera PP, Díaz-Esnal B, Laurà R (2003) Age-Related Changes in the Avian Primary Lymphoid Organs (Thymus and Bursa of Fabricius). *Microscopy Research and Technique* **62**, 482–487.

Cooper MD, Raymond DA, Peterson RD, South MA, Good RA (1966) The functions of the thymus system and the bursa system in the chicken. *The Journal of experimental medicine* **123**, 75–102.

Cosgrove AS (1962) An Apparently New Disease of Chickens: Avian

Nephrosis. *Avian Diseases* **6**, 385.

Costa B Da, Chevalier C, Henry C, Huet J-C, Petit S, Lepault J, Boot H, Delmas B (2002) The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology* **76**, 2393–402.

Coulibaly F, Chevalier C, Gutsche I, Pous J, Navaza J, Bressanelli S, Delmas B, Rey FA (2005) The birnavirus crystal structure reveals structural relationships among icosahedral viruses. *Cell* **120**, 761–72.

Darteil R, Bublot M, Laplace E, Bouquet JF, Audonnet JC, Riviè M (1995) Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. *Virology* **211**, 481–490.

Delgui L, Oña A, Gutiérrez S, Luque D, Navarro A, Castón JR, Rodríguez JF (2009) The capsid protein of infectious bursal disease virus contains a functional alpha 4 beta 1 integrin ligand motif. *Virology* **386**, 360–72.

Delmas B, Attoui H, Ghosh S, Malik YS, Mundt E, Vakharia VN (2019) ICTV virus taxonomy profile: Birnaviridae. *Journal of General Virology* **100**, 5–6.

Domanska K, Mato T, Rivallan G, Smietanka K, Minta Z, Boisseson C de, Toquin D, Lomniczi B, Palya V, Eterradossi N (2004) Antigenic and genetic diversity of early European isolates of Infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of Infectious bursal disease virus revisited? *Archives of Virology* **149**, 465–80.

Dormitorio T, Giambrone J, Duck L (1997) Sequence comparisons of the variable VP2 region of eight infectious bursal disease virus isolates. - PubMed - NCBI. *Avian Disease* **41**, 36–44.

Eterradossi N, Gauthier C, Reda I, Comte S, Rivallan G, Toquin D, Boisséson C

de, Lamandé J, Jestin V, Morin Y, Cazaban C, Borne P (2004) Extensive antigenic changes in an atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. *Avian Pathology* **33**, 423–431.

Eterradossi N, Picault JP, Drouin P, Guittet M, L'Hospitalier R, Bennejean G (1992) Pathogenicity and preliminary antigenic characterization of six infectious bursal disease virus strains isolated in France from acute outbreaks. *Journal of veterinary medicine. Series B* **39**, 683–91.

Eterradossi N, Saif YM (2013) Infectious Bursal Disease. In: *Diseases of Poultry* (eds. Swayne D, Glisson J, McDougald L, Nolan L, Suarez D, Nair V). Blackwell Publishing, Iowa, p. 1394.

Eterradossi N, Toquin D, Rivallan G, Guittet M (1997) Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. *Archives of virology* **142**, 255–70.

Fabio J Di, Rossini LI, Eterradossi N, Toquin MD, Gardin Y (1999) European-like pathogenic infectious bursal disease viruses in Brazil. *The Veterinary record* **145**, 203–4.

Fadly AM, Nazerian K (1983) Pathogenesis of infectious bursal disease in chickens infected with virus at various ages. *Avian diseases* **27**, 714–23.

Fagbohun O, Owoade A, Oluwayelu D, Olayemi F (2000) Serological survey of infectious bursal diseases virus antibodies in cattle egrets, pigeons and Nigerian laughing doves. *African Journal of Biomedical Research* **3**, 191–192.

Fahey KJ, Crooks JK, Fraser RA (1987) Assessment by ELISA of passively acquired protection against infectious bursal disease virus in chickens. *Australian veterinary journal* **64**, 203–207.

Faragher JT, Allan WH, Wyeth PJ (1974) Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. *The Veterinary record* **95**, 385–8.

Firth GA (1974) Occurrence of an infectious bursal syndrome within an Australian poultry flock. *Australian Veterinary Journal* **50**, 128–130.

Fraga AP de, Gräf T, Coltro VP, Ikuta N, Fonseca ASK, Majó N, Lunge VR (2019) Phylodynamic analyses of Brazilian antigenic variants of infectious bursal disease virus. *Infection, Genetics and Evolution* **73**, 159–166.

Galloux M, Libersou S, Morellet N, Bouaziz S, Costa B Da, Ouldali M, Lepault J, Delmas B (2007) Infectious bursal disease virus, a non-enveloped virus, possesses a capsid-associated peptide that deforms and perforates biological membranes. *Journal of Biological Chemistry* **282**, 20774–20784.

Garriga D, Querol-Audí J, Abaitua F, Saugar I, Pous J, Verdaguer N, Castón JR, Rodriguez JF (2006) The 2.6-Angstrom structure of infectious bursal disease virus-derived T=1 particles reveals new stabilizing elements of the virus capsid. *Journal of virology* **80**, 6895–905.

Ghorashi SA, O'Rourke D, Ignjatovic J, Noormohammadi AH (2011) Differentiation of infectious bursal disease virus strains using real-time RT-PCR and high resolution melt curve analysis. *Journal of Virological Methods* **171**, 264–71.

Giambrone JJ (1979) Effects of early infectious bursal disease virus infection on immunity to Newcastle disease in adult chickens. *Poultry science* **58**, 794–798.

Giambrone JJ, Clay RP (1986) Evaluation of the Immunogenicity, Stability, Pathogenicity, and Immunodepressive Potential of Four Commercial Live Infectious Bursal Disease Vaccines. *Poultry Science* **65**, 1287–1290.

Giambrone JJ, Dormitorio T, Brown T (2001) Safety and efficacy of in ovo administration of infectious bursal disease viral vaccines. *Avian diseases* **45**, 144–8.

Gimenez MC, Rodríguez Aguirre JF, Colombo MI, Delgui LR (2015) Infectious bursal disease virus uptake involves macropinocytosis and trafficking to early endosomes in a Rab5-dependent manner. *Cellular microbiology* **17**, 988–1007.

Gimenez MC, Zanetti FA, Terebiznik MR, Colombo MI, Delgui LR (2018) Infectious Bursal Disease Virus Hijacks Endosomal Membranes as the Scaffolding Structure for Viral Replication. *Journal of Virology* **92**.

Gomes AD, Abreu JT, Redondo RAF, Martins NRS, Resende JS, Resende M (2005) Genotyping of infectious bursal disease virus strains by restriction fragment length polymorphism analysis of the VP1, VP2, and VP3 genes. *Avian diseases* **49**, 500–6.

Haddad EE, Whitfill CE, Avakian AP, Ricks CA, Andrews PD, Thoma JA, Wakenell PS (1997) Efficacy of a Novel Infectious Bursal Disease Virus Immune Complex Vaccine in Broiler Chickens. *Avian Diseases* **41**, 882.

He CQ, Ma LY, Wang D, Li GR, Ding NZ (2009) Homologous recombination is apparent in infectious bursal disease virus. *Virology* **384**, 51–58.

He Z, Chen X, Fu M, Tang J, Li X, Cao H, Wang Y, Zheng SJ (2018) Infectious bursal disease virus protein VP4 suppresses type I interferon expression via inhibiting K48-linked ubiquitylation of glucocorticoid-induced leucine zipper (GILZ). *Immunobiology* **223**, 374–382.

Heine HG, Boyle DB (1993) Infectious bursal disease virus structural protein VP 2 expressed by a fowlpox virus recombinant confers protection against disease in chickens. *Archives of Virology* **131**, 277–292.

Hernández M, Banda A, Hernández D, Panzera F, Pérez R (2006) Detection of very virulent strains of infectious bursal disease virus (vvIBDV) in commercial broilers from Uruguay. *Avian diseases* **50**, 624–31.

Hernández M, Tomás G, Marandino A, Iraola G, Maya L, Mattion N, Hernández D, Villegas P, Banda A, Panzera Y, Pérez R (2015) Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. *Avian Pathology* **44**, 212–21.

Higashihara M, Saijo K, Fujisaki Y, Matumoto M (1991) Immunosuppressive effect of infectious bursal disease virus strains of variable virulence for chickens. *Veterinary microbiology* **26**, 241–8.

Hiraga M, Nunoya T, Otaki Y, Tajima M, Saito T, Nakamura T (1994) Pathogenesis of Highly Virulent Infectious Bursal Disease Virus Infection in Intact and Bursectomized Chickens. *Journal of Veterinary Medical Science* **56**, 1057–1063.

Hitchner SB (1970) Infectivity of infectious bursal disease virus for embryonating eggs. *Poultry science* **49**, 511–516.

Hollmén T, Franson JC, Docherty DE, Kilpi M, Hario M, Creekmore LH, Petersen MR (2000) Infectious Bursal Disease Virus Antibodies in Eider Ducks and Herring Gulls. *The Condor* **102**, 688–691.

Hon C-C, Lam T-Y, Drummond A, Rambaut A, Lee Y-F, Yip C-W, Zeng F, Lam P-Y, Ng PTW, Leung FCC (2006) Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. *Journal of virology* **80**, 8503–9.

Huang Z, Elankumaran S, Yunus AS, Samal SK (2004) A Recombinant Newcastle Disease Virus (NDV) Expressing VP2 Protein of Infectious Bursal

Disease Virus (IBDV) Protects against NDV and IBDV. *Journal of Virology* **78**, 10054–10063.

Ide PR, Schulte-Nordholt JA, Dewitt WF, Smith JD (1978) Broiler-breeder vaccination against infectious bursal disease and persistence of maternal antibody in progeny. *The Canadian veterinary journal* **19**, 123–7.

Ignjatovic J, Sapats S (2002) Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. *Australian veterinary journal* **80**, 689–94.

Irigoyen N, Castón JR, Rodríguez JF (2012) Host proteolytic activity is necessary for infectious bursal disease virus capsid protein assembly. *The Journal of biological chemistry* **287**, 24473–82.

Ismail N, Saif Y (1988) Immunological studies on infectious bursal disease viruses in chickens. In: *Conference of Research Workers in Animal Disease*. Chicago, p. 344.

Ismail NM, Fadly AM, Chang TS (1987) Effect of bursal cell number on the pathogenesis of infectious bursal disease in chickens. *Avian diseases* **31**, 546–55.

Ismail NM, Saif YM, Moorhead PD (1988a) Lack of pathogenicity of five serotype 2 infectious bursal disease viruses in chickens. *Avian Diseases* **32**, 757–9.

Ismail NM, Saif YM, Moorhead PD (1988b) Lack of Pathogenicity of Five Serotype 2 Infectious Bursal Disease Viruses in Chickens. *Avian Diseases* **32**, 757.

Ismail NM, Saif YM, Wigle WL, Havenstein GB, Jackson C (1990) Infectious bursal disease virus variant from commercial Leghorn pullets. *Avian diseases* **34**, 141–5.

Iván J, Velhner M, Ursu K, Germán P, Mató T, Drén CN, Mészáros J (2005) Delayed vaccine virus replication in chickens vaccinated subcutaneously with an immune complex infectious bursal disease vaccine: Quantification of vaccine virus by real-time polymerase chain reaction. *Canadian Journal of Veterinary Research* **69**, 135–142.

Jackwood DJ, Gough RE, Sommer SE (2005) Nucleotide and amino acid sequence analysis of a birnavirus isolated from penguins. *The Veterinary record* **156**, 550–2.

Jackwood DJ, Saif YM (1983) Prevalence of antibodies to infectious bursal disease virus serotypes I and II in 75 Ohio chicken flocks. *Avian diseases* **27**, 850–4.

Jackwood DJ, Saif YM, Hughes JH (1982) Characteristics and Serologic Studies of Two Serotypes of Infectious Bursal Disease Virus in Turkeys. *Avian Diseases* **26**, 871.

Jackwood DJ, Saif YM, Moorhead PD, Bishop G (1984) Failure of two serotype II infectious bursal disease viruses to affect the humoral immune response of turkeys. *Avian diseases* **28**, 100–16.

Jackwood DJ, Schat KA, Michel LO, Wit S de (2018) A proposed nomenclature for infectious bursal disease virus isolates. *Avian Pathology* **47**, 576–584.

Jackwood DJ, Sreedevi B, LeFever LJ, Sommer-Wagner SE (2008) Studies on naturally occurring infectious bursal disease viruses suggest that a single amino acid substitution at position 253 in VP2 increases pathogenicity. *Virology* **377**, 110–116.

Jungmann A, Nieper H, Müller H (2001) Apoptosis is induced by infectious bursal disease virus replication in productively infected cells as well as in antigen-negative cells in their vicinity. *Journal of General Virology* **82**, 1107–

1115.

Kasanga CJ, Yamaguchi T, Munang'andu HM, Ohya K, Fukushi H (2013) Genomic sequence of an infectious bursal disease virus isolate from Zambia: Classical attenuated segment B reassortment in nature with existing very virulent segment A. *Archives of Virology*.

Kibenge FSB, Jackwood DJ, Mercado CC (1990) Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. *Journal of General Virology* **71**, 569–577.

Kim I-J, Gagic M, Sharma JM (1999) Recovery of Antibody-Producing Ability and Lymphocyte Repopulation of Bursal Follicles in Chickens Exposed to Infectious Bursal Disease Virus. *Avian Diseases* **43**, 401.

Kong LL, Omar AR, Hair Bejo M, Ideris A, Tan SW (2009) Development of SYBR green I based one-step real-time RT-PCR assay for the detection and differentiation of very virulent and classical strains of infectious bursal disease virus. *Journal of Virological Methods* **161**, 271–279.

Kutyavin I V, Afonina IA, Mills A, Gorn V V, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J (2000) 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research* **28**, 655–61.

Lasher HN, Davis VS (1997) History of Infectious Bursal Disease in the U.S.A.: The First Two Decades. *Avian Diseases* **41**, 11.

Lee CC, Ko TP, Chou CC, Yoshimura M, Doong SR, Wang MY, Wang AHJ (2006) Crystal structure of infectious bursal disease virus VP2 subviral particle at 2.6 Å resolution: Implications in virion assembly and immunogenicity. *Journal of Structural Biology* **155**, 74–86.

Lejal N, Costa BD, Huet JC, Delmas B, Costa B Da (2000) Role of Ser-652 and

Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology* **81**, 983–92.

Letzel T, Coulibaly F, Rey FA, Delmas B, Jagt E, Loon AAMW van, Mundt E (2007) Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *Journal of virology* **81**, 12827–35.

Ley D, Storm N, Bickford A, Yamamoto R (1979) An infectious bursal disease virus outbreak in 14- and 15-week-old chickens. - PubMed - NCBI. *vian Diseases* **23**, 235–40.

Li K, Courtillon C, Guionie O, Allée C, Amelot M, Qi X, Gao Y, Wang X, Eterradossi N (2015) Genetic, antigenic and pathogenic characterization of four infectious bursal disease virus isolates from China suggests continued evolution of very virulent viruses. *Infection, Genetics and Evolution* **30**, 120–127.

Li Z, Wang Y, Xue Y, Li X, Cao H, Zheng SJ (2012) Critical role for voltage-dependent anion channel 2 in infectious bursal disease virus-induced apoptosis in host cells via interaction with VP5. *Journal of virology* **86**, 1328–38.

Lin T-W, Lo C-W, Lai S-Y, Fan R-J, Lo C-J, Chou Y -m., Thiruvengadam R, Wang AH-J, Wang M-Y (2007) Chicken Heat Shock Protein 90 Is a Component of the Putative Cellular Receptor Complex of Infectious Bursal Disease Virus. *Journal of Virology* **81**, 8730–8741.

Liu M, Vakharia VN (2006) Nonstructural protein of infectious bursal disease virus inhibits apoptosis at the early stage of virus infection. *Journal of virology* **80**, 3369–77.

Lombardo E, Maraver A, Castón JR, Rivera J, Fernández-Arias A, Serrano A,

Carrascosa JL, Rodriguez JF (1999) VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *Journal of virology* **73**, 6973–83.

Lombardo E, Maraver A, Espinosa I, Fernández-Arias A, Rodriguez JF (2000) VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. *Virology* **277**, 345–357.

Lu Z, Zhang L, Wang N, Chen Y, Gao L, Wang Y, Gao H, Gao Y, Li K, Qi X, Wang X (2015) Naturally occurring reassortant infectious bursal disease virus in northern China. *Virus Research* **203**, 92–95.

Luo J, Zhang H, Teng M, Fan J-M, You L-M, Xiao Z-J, Yi M-L, Zhi Y-B, Li X-W, Zhang G-P (2010) Surface IgM on DT40 cells may be a component of the putative receptor complex responsible for the binding of infectious bursal disease virus. *Avian pathology* **39**, 359–65.

Lupini C, Giovanardi D, Pesente P, Bonci M, Felice V, Rossi G, Morandini E, Cecchinato M, Catelli E (2016) A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. *Avian Pathology* **45**, 458–464.

Luque D, Saugar I, Rejas MT, Carrascosa JL, Rodríguez JF, Castón JR (2009) Infectious Bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *Journal of Molecular Biology* **386**, 891–901.

MacKenzie MA, Spradbrow PB (1983) A study of the epidemiology of infectious bursal disease in poultry in Queensland, 1976–1979. *Australian Veterinary Journal* **60**, 52–54.

Maraver A, Ona A, Abaitua F, Gonzalez D, Clemente R, Ruiz-Diaz JA, Caston

JR, Pazos F, Rodriguez JF (2003) The Oligomerization Domain of VP3, the Scaffolding Protein of Infectious Bursal Disease Virus, Plays a Critical Role in Capsid Assembly. *Journal of Virology* **77**, 6438–6449.

Martin AM, Fallacara F, Barbieri I, Tosi G, Rivallan G, Eterradossi N, Ceruti R, Cordioli P (2007) Genetic and antigenic characterization of infectious bursal disease viruses isolated in Italy during the period 2002-2005. *Avian Diseases* **51**, 863–72.

Mata CP, Mertens J, Fontana J, Luque D, Allende-Ballestero C, Reguera D, Trus BL, Steven AC, Carrascosa JL, Castón JR (2018) The RNA-Binding Protein of a Double-Stranded RNA Virus Acts like a Scaffold Protein. *Journal of virology* **92**.

Mazariegos LA, Lukert PD, Brown J (1990) Pathogenicity and immunosuppressive properties of infectious bursal disease strains. *Avian diseases* **34**, 203–8.

McFerran JB, McNulty MS, McKillop ER, Connor TJ, McCracken RM, Collins DS, Allan GM (1980) Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. *Avian pathology* **9**, 395–404.

McNulty MS, Saif YM (1988) Antigenic relationship of non-serotype 1 turkey infectious bursal disease viruses from the United States and United Kingdom. *Avian diseases* **32**, 374–5.

Meulemans G, Halen P (1982) Efficacy of some disinfectants against infectious bursal disease virus and avian reovirus. *Veterinary Record* **111**, 412–413.

Michel LO, Jackwood DJ (2017) Classification of infectious bursal disease virus into genogroups. *Archives of Virology* **162**, 3661–3670.

Minta Z, Roszkowski J, Karczewski W, Kozaczynski W (1985a) Properties of

indigenous strains of infectious bursal disease virus. II. Pathogenicity. *Bulletin of the Veterinary Institute in Pulawy* **28**–**29**, 67–73.

Minta Z, Roszkowski J, Karczewski W, Kozaczynski W (1985b) Properties of indigenous strains of infectious bursal disease virus. III. Immunosuppressive effect. *Bulletin of the Veterinary Institute in Pulawy* **28**–**29**, 73–76.

Molini U, Aikukutu G, Kabajani J, Khaiseb S, Cattoli G (2019) Molecular characterisation of infectious bursal disease virus in Namibia, 2017. *Onderstepoort Journal of Veterinary Research* **86**.

Müller H, Mundt E, Eterradossi N, Islam MR (2012) Current status of vaccines against infectious bursal disease. *Avian Pathology* **41**, 133–139.

Müller R, Käufer I, Reinacher M, Weiss E (1979) Immunofluorescent Studies of Early Virus Propagation after Oral Infection with Infectious Bursal Disease Virus (IBDV). *Zentralblatt für Veterinärmedizin Reihe B* **26**, 345–352.

Mundt E, Beyer J, Müller H (1995) Identification of a novel viral protein in infectious bursal disease virus-infected cells. *Journal of General Virology* **76**, 437–443.

Mundt E, Köllner B, Kretzschmar D (1997) VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. *Journal of virology* **71**, 5647–51.

Mundt E, Müller H (1995) Complete nucleotide sequences of 5′- and 3′-noncoding regions of both genome segments of different strains of infectious bursal disease virus. *Virology* **209**, 10–18.

Nader K, Hardt O, Lanius R (2013) Systemic and local antibody responses to infectious bronchitis virus in chickens inoculated with infectious bursal disease virus and control chickens. *Dialogues in clinical neuroscience* **15**, 475–86.

Nick H, Cursiefen D, Becht H (1976) Structural and growth characteristics of infectious bursal disease virus. *Journal of virology* **18**, 227–34.

Nielsen OL, Sørensen P, Hedemand JE, Laursen SB, Jørgensen PH (1998) Inflammatory response of different chicken lines and B haplotypes to infection with infectious bursal disease virus. *Avian Pathology* **27**, 181–189.

Noor M, Mahmud MS, Ghose PR, Roy U, Nooruzzaman M, Chowdhury EH, Das PM, Islam MR, Müller H (2014) Further evidence for the association of distinct amino acid residues with in vitro and in vivo growth of infectious bursal disease virus. *Archives of Virology* **159**, 701–709.

Nouën C Le, Rivallan G, Toquin D, Darlu P, Morin Y, Beven V, Boisseson C de, Cazaban C, Comte S, Gardin Y, Eterradossi N (2006) Very virulent infectious bursal disease virus: Reduced pathogenicity in a rare natural segment-B-reassorted isolate. *Journal of General Virology* **87**, 209–216.

Nunoya T, Otaki Y, Tajima M, Hiraga M, Saito T (1992) Occurrence of Acute Infectious Bursal Disease with High Mortality in Japan and Pathogenicity of Field Isolates in Specific-Pathogen-Free Chickens. *Avian Diseases* **36**, 597.

Ogawa M, Wakuda T, Yamaguchi T, Murata K, Setiyono A, Fukushi H, Hirai K (1998) Seroprevalence of infectious bursal disease virus in free-living wild birds in Japan. *The Journal of veterinary medical science* **60**, 1277–9.

Oña A, Luque D, Abaitua F, Maraver A, Castón JR, Rodríguez JF (2004) The C-terminal domain of the pVP2 precursor is essential for the interaction between VP2 and VP3, the capsid polypeptides of infectious bursal disease virus. *Virology* **322**, 135–142.

Organización Mundial de Sanidad Animal (2018) Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres 2019. Bursitis infecciosa (Enfermedad de Gumboro).

Pan J, Lin L, Tao YJ (2009) Self-guanylylation of birnavirus VP1 does not require an intact polymerase activity site. *Virology* **395**, 87–96.

Parkhurst RT (1964) On-the-Farm Studies of Gumboro Disease in Broilers. *Avian Diseases* **8**, 584.

Peck KM, Lauring AS (2018) Complexities of Viral Mutation Rates. *Journal of Virology* **92**.

Perelman B, Heller ED (1983) The effect of infectious bursal disease virus on the immune system of turkeys. *Avian diseases* **27**, 66–76.

Petek M, D'Aprile PN, Cancellotti F (1973) Biological and physico-chemical properties of the infectious bursal disease virus (ibdv). *Avian Pathology* **2**, 135–152.

Peters MA, Lin TL, Wu CC (2005) Real-time RT-PCR differentiation and quantitation of infectious bursal disease virus strains using dual-labeled fluorescent probes. *Journal of Virological Methods* **127**, 87–95.

Pikuła A, Lisowska A, Jasik A, Śmietanka K (2018) Identification and assessment of virulence of a natural reassortant of infectious bursal disease virus. *Veterinary Research* **49**.

Poonia B, Charan S (2004) Infiltration by CD4+ and CD8+ lymphocytes in bursa of chickens infected with Infectious Bursal Disease Virus (IBDV): strain-specific differences. *Indian journal of experimental biology* **42**, 823–9.

Qi X, Gao H, Gao Y, Qin L, Wang Y, Gao L, Wang X (2009) Naturally occurring mutations at residues 253 and 284 in VP2 contribute to the cell tropism and virulence of very virulent infectious bursal disease virus. *Antiviral Research* **84**, 225–233.

Qi X, Zhang L, Chen Y, Gao L, Wu G, Qin L, Wang Y, Ren X, Gao Y, Gao H, Wang X (2013) Mutations of Residues 249 and 256 in VP2 Are Involved in the

Replication and Virulence of Infectious Bursal Disease Virus. *PLoS ONE* **8**.

Rautenschlein S, Yeh HY, Njenga MK, Sharma JM (2002) Role of intrabursal T cells in infectious bursal disease virus (IBDV) infection: T cells promote viral clearance but delay follicular recovery. *Archives of Virology* **147**, 285–304.

Rosenberger JK, Cloud SS (1985) Isolation and characterization of variant infectious bursal disease viruses. *J Am Vet Med Assoc* **189**, 357.

Rosenberger JK, Cloud SS (1989) The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian diseases* **33**, 753–9.

Rosenberger JK, Cloud SS, Gelb JJ, Odor E, Dohms JE (1985) Sentinel bird survey of Delmarva broiler flocks. In: *Proc 20th Natl Meet Poult Health Condemn*. Ocean City, MD, pp. 94–101.

Rosenberger JK, Gelb J (1978) Response to several avian respiratory viruses as affected by infectious bursal disease virus. *Avian Diseases* **22**, 95–105.

Saif Y (1984) Infectious bursal disease virus types. In: *Proc 19th Natl Meet Poult Health Condemn*. Ocean City, MD, pp. 105–107.

Samy A, Courtillon C, Briand FX, Khalifa M, Selim A, Arafa AES, Hegazy A, Eterradossi N, Soubies SM (2020) Continuous circulation of an antigenically modified very virulent infectious bursal disease virus for fifteen years in Egypt. *Infection, Genetics and Evolution* **78**.

Sánchez AB, Rodriguez JF (1999) Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology* **262**, 190–9.

Sapats SI, Ignjatovic J (2000) Antigenic and sequence heterogeneity of infectious bursal disease virus strains isolated in Australia. *Archives of virology* **145**, 773–85.

Saugar I, Luque D, Oña A, Rodríguez JF, Carrascosa JL, Trus BL, Castón JR (2005) Structural polymorphism of the major capsid protein of a double-stranded RNA virus: An amphipathic α helix as a molecular switch. *Structure* **13**, 1007–1017.

Sharma JM, Kim IJ, Rautenschlein S, Yeh HY (2000) Infectious bursal disease virus of chickens: Pathogenesis and immunosuppression. *Developmental and Comparative Immunology* **24**, 223–235.

Silva FMF, Vidigal PMP, Myrrha LW, Fietto JLR, Silva A, Almeida MR (2013) Tracking the molecular epidemiology of Brazilian Infectious bursal disease virus (IBDV) isolates. *Infection, Genetics and Evolution* **13**, 18–26.

Silva MS e, Rissi DR, Swayne DE (2016) Very Virulent Infectious Bursal Disease Virus Produces More-Severe Disease and Lesions in Specific-Pathogen-Free (SPF) Leghorns Than in SPF Broiler Chickens. *Avian Diseases* **60**, 63–66.

Skeeles J, Lukert P, Buysscher E De, Fletcher O, Brown J (1979) Infectious bursal disease viral infections. II. The relationship of age, complement levels, virus-neutralizing antibody, clotting, and lesions. *Avian Diseases* **23**, 107–17.

Snyder DB, Vakharia VN, Savage PK (1992) Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. *Archives of virology* **127**, 89–101.

Soubies SM, Courtillon C, Abed M, Amelot M, Keita A, Broadbent A, Härtle S, Kaspers B, Eterradossi N (2018) Propagation and titration of infectious bursal disease virus, including non-cell-culture-adapted strains, using *ex vivo* -stimulated chicken bursal cells. *Avian Pathology* **47**, 179–188.

Soubies SM, Courtillon C, Briand F-X, Queguiner-Leroux M, Courtois D, Amelot M, Grousson K, Morillon P, Herin J-B, Eterradossi N (2017) Identification of a European interserotypic reassortant strain of infectious

bursal disease virus. *Avian Pathology* **46**, 19–27.

Spies U, Müller H, Becht H (1987) Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Research* **8**, 127–40.

Takase K, Nonaka F, Fukuda T, Yamada S (1982) Recovery of virus from feces and tissues of chickens infected with cell-culture-adapted infectious bursal disease virus. *Nihon juigaku zasshi. The Japanese journal of veterinary science* **44**, 207–11.

Tippenhauer M, Heller DE, Weigend S, Rautenschlein S (2013) The host genotype influences infectious bursal disease virus pathogenesis in chickens by modulation of T cells responses and cytokine gene expression. *Developmental and comparative immunology* **40**, 1–10.

Tomás G, Hernández M, Marandino A, Hernández D, Techera C, Grecco S, Panzera Y, Pérez R (2015) Genome Sequence of a Distinct Infectious Bursal Disease Virus. *Genome Announcements* **3**.

Tomás G, Hernández M, Marandino A, Panzera Y, Maya L, Hernández D, Pereda A, Banda A, Villegas P, Aguirre S, Pérez R (2012) Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. *Journal of Virological Methods* **185**, 101–7.

Tomás G, Hernández M, Marandino A, Techera C, Grecco S, Hernández D, Banda A, Panzera Y, Pérez R (2017) Development of an RT-qPCR assay for the specific detection of a distinct genetic lineage of the infectious bursal disease virus. *Avian Pathology* **46**, 150–156.

Tomás G, Marandino A, Courtillon C, Amelot M, Keita A, Pikula A, Hernández M, Hernández D, Vagnozzi A, Panzera Y, Domańska-Blicharz K, Eterradossi N,

Pérez R, Soubies SM (2019) Antigenicity, pathogenicity and immunosuppressive effect caused by a South American isolate of infectious bursal disease virus belonging to the "distinct" genetic lineage. *Avian Pathology* **48**, 245–254.

Tomás G, Marandino A, Techera C, Olivera V, Perbolianachis P, Fuques E, Grecco S, Hernández M, Hernández D, Calleros L, Craig M, Panzera Y, Vagnozzi A, Pérez R (2020) Origin and global spreading of an ancestral lineage of the infectious bursal disease virus. *Transboundary and Emerging Diseases*.

Tsukamoto K, Kojima C, Komori Y, Tanimura N, Mase M, Yamaguchi S (1999) Protection of chickens against very virulent infectious bursal disease virus (IBDV) and Marek's disease virus (MDV) with a recombinant MDV expressing IBDV VP2. *Virology* **257**, 352–362.

Vakharia VN, He J, Ahamed B, Snyder DB (1994) Molecular basis of antigenic variation in infectious bursal disease virus. *Virus research* **31**, 265–73.

Vera F, Craig MI, Olivera V, Rojas F, König G, Pereda A, Vagnozzi A (2015) Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. *Archives of Virology* **160**, 1909–21.

Wang Q, Hu H, Chen G, Liu H, Wang S, Xia D, Yu Y, Zhang Y, Jiang J, Ma J, Xu Y, Xu Z, Ou C, Liu X (2019) Identification and assessment of pathogenicity of a naturally reassorted infectious bursal disease virus from Henan, China. *Poultry Science*.

Wang YS, Wang ZC, Tang YD, Shi ZL, He KW, Li Y, Hou JB, Yao HC, Fan HJ, Lu CP (2007) Comparison of four infectious bursal disease viruses isolated from different bird species. *Archives of virology* **152**, 1787–97.

Wei L, Zhu S, Ruan G, Hou L, Wang J, Wang B, Liu J (2011) Infectious bursal

disease virus-induced activation of JNK signaling pathway is required for virus replication and correlates with virus-induced apoptosis. *Virology* **420**, 156–163.

Wei Y, Li J, Zheng J, Xu H, Li L, Yu L (2006) Genetic reassortment of infectious bursal disease virus in nature. *Biochemical and Biophysical Research Communications* **350**, 277–287.

Wei Y, Yu X, Zheng J, Chu W, Xu H, Yu X, Yu L (2008) Reassortant infectious bursal disease virus isolated in China. *Virus Research* **131**, 279–282.

Westbury HA (1978) Interaction between infectious bursal disease virusand Newcastle disease virus in chickens. *Australian Veterinary Journal* **54**, 349–351.

Whitfill CE, Haddad EE, Ricks CA, Skeeles JK, Newberry LA, Beasley JN, Andrews PD, Thoma JA, Wakenell PS (1995) Determination of optimum formulation of a novel infectious bursal disease virus (IBDV) vaccine constructed by mixing bursal disease antibody with IBDV. *Avian diseases* **39**, 687–99.

Winterfield R, Hitchner S, Appleton G, Cosgrove A (1962) Avian nephrosis, nephritis and Gumboro disease. *L&M News Views* **3**, 103.

Winterfield RW, Hitchner SB (1962) Etiology of an infectious nephritis-nephrosis syndrome of chickens. *American journal of veterinary research* **23**, 1273–9.

Withers DR, Davison TF, Young JR (2006) Diversified bursal medullary B cells survive and expand independently after depletion following neonatal infectious bursal disease virus infection. *Immunology* **117**, 558–565.

Wu CC, Rubinelli P, Lin TL (2007) Molecular detection and differentiation of infectious bursal disease virus. *Avian Diseases* **51**, 515–26.

Wu Y, Hong L, Ye J, Huang Z, Zhou J (2009) The VP5 protein of infectious bursal disease virus promotes virion release from infected cells and is not involved in cell death. *Archives of Virology* **154**, 1873–82.

Wyeth PJ, Cullen GA (1978) Transmission of immunity from inactivated infectious bursal disease oil-emulsion vaccinated parent chickens to their chicks. *The Veterinary record* **102**, 362–363.

Yilmaz A, Turan N, Bayraktar E, Gurel A, Cizmecigil UY, Aydin O, Bamac OE, Cecchinato M, Franzo G, Tali HE, Cakan B, Savic V, Richt JA, Yilmaz H (2019) Phylogeny and evolution of infectious bursal disease virus circulating in Turkish broiler flocks. *Poultry Science* **98**, 1976–1984.

Zhao Y, Aarnink AJA, Cambra-Lopez M, Fabri T (2013) Viral shedding and emission of airborne infectious bursal disease virus from a broiler room. *British poultry science* **54**, 87–95.

*Avian Pathology*, 2015
http://dx.doi.org/10.1080/03079457.2015.1025696

Taylor & Francis
Taylor & Francis Group

**ORIGINAL ARTICLE**

# Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage

Martín Hernández[1], Gonzalo Tomás[1], Ana Marandino[1], Gregorio Iraola[1], Leticia Maya[1], Nora Mattion[2], Diego Hernández[1], Pedro Villegas[3], Alejandro Banda[4], Yanina Panzera[1], and Ruben Pérez[1*]

[1]*Sección Genética Evolutiva, Departamento de Biología Animal, Instituto de Biología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay,* [2]*Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. César Milstein, Buenos Aires, Argentina,* [3]*Poultry Diagnostic and Research Center, College of Veterinary Medicine, The University of Georgia, Athens, GA, USA, and* [4]*Poultry Research and Diagnostic Laboratory, College of Veterinary Medicine, Mississippi State University, Pearl, MS, USA*



Infectious bursal disease virus (IBDV) is one of the most concerning health problems for world poultry production. IBDVs comprise four well-defined evolutionary lineages known as classic (c), classic attenuated (ca), variant (va) and very virulent (vv) strains. Here, we characterized IBDVs from South America by the genetic analysis of both segments of the viral genome. Viruses belonging to c, ca and vv strains were unambiguously classified by the presence of molecular markers and phylogenetic analysis of the hypervariable region of the *vp2* gene. Notably, the majority of the characterized viruses (9 out of 15) could not be accurately assigned to any of the previously described strains and were then denoted as distinct (d) IBDVs. These dIBDVs constitute an independent evolutionary lineage that also comprises field IBDVs from America, Europe and Asia. The hypervariable VP2 sequence of dIBDVs has a unique and conserved molecular signature (272T, 289P, 290I and 296F) that is a diagnostic character for classification. A discriminant analysis of principal components (DAPC) also identified the dIBDVs as a cluster of genetically related viruses separated from the typical strains. DAPC and genetic distance estimation indicated that the dIBDVs are one of the most genetically divergent IBDV lineages. The *vp1* gene of the dIBDVs has non-vvIBDV markers and unique nucleotide and amino acid features that support their divergence in both genomic segments. The present study suggests that the dIBDVs comprise a neglected, highly divergent lineage that has been circulating in world poultry production since the early time of IBDV emergence.

## Introduction

Infectious bursal disease is an acute, highly contagious viral disease that affects the immune system of young chickens and causes major economic losses to the poultry industry worldwide. The aetiologic agent is the infectious bursal disease virus (IBDV) belonging to the genus *Avibirnavirus* within the family *Birnaviridae*. IBDV is a non-enveloped icosahedral virus with a double-stranded RNA genome containing two segments (Müller *et al.*, 1979). Segment A (3.3 kb) has two partially overlapping open reading frames. Open reading frame A-1 encodes a polyprotein that is autocatalytically cleaved to yield the immature outer capsid protein pVP2, the viral protease VP4 and the ribonucleoprotein VP3 (Sánchez & Rodriguez, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002; Luque *et al.*, 2009). The pVP2 protein is further processed to yield the mature VP2, the immunodominant antigen of IBDV (Fahey *et al.*, 1991). Open reading frame A-2 encodes VP5, a non-structural protein involved in virion release from infected cells (Wu *et al.*, 2009). Genomic segment B (2.9 kb) encodes the RNA-dependent RNA polymerase VP1 (Spies *et al.*, 1987).

Two IBDV serotypes (1 and 2) are currently characterized by cross-neutralization assays. Serotype 2 has not been associated with disease in any avian species, while serotype 1 comprises all the known pathogenic IBDVs (McFerran *et al.*, 1980; Jackwood *et al.*, 1985). First outbreaks of the pathogenic serotype 1 occurred more than 50 years ago in Gumboro (Delaware, USA) (Cosgrove, 1962). The associated viruses, currently referred as classic (c) IBDVs, caused high morbidity and mortality in the affected broiler flocks. Vaccines against cIBDVs were rapidly developed and introduced in the routine vaccination schedules, reducing considerably the mortality rates and partially controlling the disease (Snedeker *et al.*, 1967; Edgar & Cho, 1976). This epidemiological scenario changed in the late 1980s with the emergence of the variant (va) and very virulent (vv) strains.

The vaIBDVs were characterized by an antigenic drift caused by point mutations affecting the neutralizing epitopes

*To whom correspondence should be addressed: E-mail: rperez@fcien.edu.uy
(*Received 19 December 2014; accepted 24 February 2015*)

of VP2 (Ismail *et al.*, 1990; Vakharia *et al.*, 1994). These strains emerged in the North American continent and were characterized by causing B-lymphocyte depletion without eliciting an inflammatory response or clinical signs of disease (Rosales *et al.*, 1989; Sharma *et al.*, 1989). Although the vaIBDVs were clearly serotype 1 viruses, they had enough antigenic differences to evade the immunity induced by classic-type vaccines.

Almost simultaneously with the vaIBDV emergence, vvIBDVs emerged in Europe and caused a very severe form of the disease with unusual high mortality (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). These vvIBDVs were antigenically similar to the cIBDVs, but they were able to breakthrough the existing level of maternally derived protection (van den Berg *et al.*, 1996).

The three IBDV strains (c, va and vv) currently have a global distribution and occur in most countries with a developed poultry industry. Isolated IBDVs with different traits than the traditional strains have also been sporadically reported through the years in different parts of the world (Ikuta *et al.*, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007). These IBDVs have been generally considered atypical isolates or variants that have evolved in restricted geographic regions or during short periods of time under particular conditions.

An accurate identification of the field variants circulating in the poultry production has been essential to understand the epidemiology and control the disease (van den Berg, 2000). Genetic analysis has been the most widely used technique to characterize and classify IBDVs (Cao *et al.*, 1998; Banda & Villegas, 2004; Hernández *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Yamaguchi *et al.*, 2007; Tomás *et al.*, 2012). Studies have been mainly focused on the hypervariable domain of VP2 (hvVP2) because it codes for the most important immunogenic regions of the protein (Azad *et al.*, 1987; Bayliss *et al.*, 1990).

Virus classification can be performed by identifying nucleotide or amino acid residues specific for each IBDV strain. These strain markers are determined by molecular typing techniques (e.g., restriction fragment length polymorphisms, real-time polymerase chain reaction, PCR) or sequencing. A more comprehensive classification is achieved by phylogenetic analysis using mainly the hvVP2 sequence; viruses are classified according with their association with reference strains in a well-supported clade that share a single common ancestor, i.e., evolutionary lineage (Jackwood & Sommer-Wagner, 2007; Yamaguchi *et al.*, 2007; Kim *et al.*, 2010; Islam *et al.*, 2012). Phylogenetic trees using the hvVP2 region consistently recover the clades corresponding to the three IBDV strains (c, va and vv), and the clade composed of attenuated classic vaccine strains, usually denoted as "classic attenuated" (ca) IBDVs (Martin *et al.*, 2007; Wu *et al.*, 2007; Xia *et al.*, 2008; Kim *et al.*, 2010).

In addition to sequences of segment A, the analysis of the *vp1* gene of segment B is useful in providing complementary genetic information for more precise characterization. By studying both segments, it is possible to analyse their coevolution, to detect the occurrence of natural reassortants and to determine the role of VP1 in pathogenicity (Le Nouën *et al.*, 2006; Escaffre *et al.*, 2013). VP1 phylogeny resolves a lineage corresponding to the vvIBDV strain and a non-vvIBDV clade constituted by the remaining strains and serotype 2 viruses (Hon *et al.*, 2006).

The present study presents genetic comparative analyses of Uruguayan and Argentine field viruses with global IBDVs and the molecular characterization of a worldwide-spread genetic lineage divergent from the traditional IBDV strains.

## Materials and Methods

**Viruses.** Bursal samples were collected during 2007–2012 from Uruguayan and Argentine broiler flocks. Samples came from chickens with clinical signs of immunodeficiency frequently associated with respiratory or urinary infections. Each sample was stored at –80°C until analysis. IBDV presence was confirmed in 15 samples (Table 1) with real-time PCR, as previously described (Tomás *et al.*, 2012).

**PCR amplifications.** PCR amplifications of a 643-bp fragment encompassing the hvVP2 region and a 594-bp fragment of the *vp1* gene were performed using previously described primers and conditions (Hernández *et al.*, 2006). An Applied Biosystems 2720 PCR Thermal Cycler was used for the amplification reaction. PCR products were assayed on 0.8% agarose gels stained with ethidium bromide, and purified using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK), and sequenced bi-directionally using an ABI prism 377-Perkin Elmer automated sequencer.

**Phylogenetic inferences.** Sequence homologies were first analysed using the nucleotide BLAST algorithm with GenBank database (http://www.ncbi.nlm.nih.gov). The Uruguayan and Argentine viruses were further compared with reference and representative IBDVs. Phylogenetic analyses included sequences encompassing 374 bp of hvVP2 coding region from position 795 to 1168, and a 534-bp fragment from nucleotide positions 322 to 855 of the *vp1* gene (genome numbering according to D6948 segment A and B, AF240686 and AF240687, respectively).

Sequences were aligned using MAFFT (Katoh *et al.*, 2002), and the best-fit model of nucleotide substitution was selected under the Akaike information criterion and Bayesian information criterion as implemented in jModelTest (Posada, 2008). Maximum-likelihood trees, with approximate likelihood ratio test for internal nodes support, were inferred using PhyML (Guindon & Gascuel, 2003). Phylogenetic trees were visualized and edited with TreeGraph2 (Stöver & Müller, 2010).

**Discriminant analysis of principal components and between-group distance.** The discriminant analysis of principal components (DAPC) multivariate method (Jombart *et al.*, 2010), used to infer the genetic structure of populations coupling classical principal components analysis and discriminant analysis, was performed with the VP2 nucleotide sequences used in the phylogenetic analysis and additional representatives of the different IBDV

**Table 1.** *Argentine and Uruguayan IBDV characterized in this study.*

| Name | Origin | Type | Accession[a] |
|---|---|---|---|
| AR-A1 | Argentina | vvIBDV | KM659881 |
| AR-C7 | Argentina | cIBDV | KM659882 |
| UY-130D | Uruguay | caIBDV | KM659884 |
| UY-131D | Uruguay | caIBDV | KM659885 |
| UY-139D | Uruguay | caIBDV | KM659886 |
| UY-01/09 | Uruguay | caIBDV | KM659883 |
| AR-C1 | Argentina | dIBDV | KM659888 |
| AR-C3 | Argentina | dIBDV | KM659889 |
| UY-42/07 | Uruguay | dIBDV | KM659892 |
| UY-04/09 | Uruguay | dIBDV | KM659895 |
| UY-06/10 | Uruguay | dIBDV | KM659890 |
| UY-07/10A | Uruguay | dIBDV | KM659891 |
| UY-171101 | Uruguay | dIBDV | KM659887 |
| UY-421101 | Uruguay | dIBDV | KM659893 |
| UY-221201 | Uruguay | dIBDV | KM659894 |

[a]Accession numbers correspond to VP2 coding sequences (hypervariable region). The vp1 sequences of samples UY-07/10A, UY-06/10; UY-221201 and UY-04/09 were deposited under the accession numbers KP274850-3, respectively.

strains. This method was applied using pre-defined groups and 75 principal components were retained and inputted to discriminant analysis. The analysis of the nucleotide sequences was implemented using *adegenet* package library of R statistical environment (R Development Core Team, 2013). Additionally, net between-groups mean distances were calculated as implemented in MEGA5.1 (Tamura *et al.*, 2011).

**Accession numbers.** The hvVP2 coding sequences and the partial sequences of the *vp1* gene were deposited under the GenBank accession numbers KM659881-95 and KP274850-3, respectively.

## Results

An initial classification of the Argentine and Uruguayan IBDVs was performed by identifying amino acid markers of the hvVP2 coding region and comparing their nucleotide sequences with reference strains (c, ca, va and vv) (Figure 1). Six samples were straightforward to classify as belonging to traditional IBDV strains, but nine samples had singular genetic and amino acid sequences and were here denoted as distinct (d) IBDVs.

**Very virulent infectious bursal disease viruses.** Argentine AR-A1 has the vvIBDV signature 222A, 256I, 294I and 299S. This virus shows a maximum nucleotide similarity (99.8%) with the vv reference strain D6948, and high similarity with the Argentine hypervirulent isolates LD-847-04 and LD9569 (99.3% and 98.8%, respectively) and with previously reported Uruguayan vvIBDVs (98.8%).

**Classic infectious bursal disease viruses.** Argentine AR-C7 has the markers 263F and 312V observed in STC, one of the c reference isolates. Comparative nucleotide sequence analysis shows 100% nucleotide similarity with the cIBDVs HPR-2 and A-BH83 from the USA and Brazil, respectively, and has 99.8% of similarity with STC.

**Classic attenuated infectious bursal disease viruses.** Four Uruguayan viruses (UY-130D, UY-131D, UY-139D and UY-01/09) have the amino acid composition characteristic of caIBDVs. With the exception of UY-01/09, which shows the residue 253N, the remaining three isolates contain the signature 253H, 279N and 284T, typical of the classic vaccine viruses. The highest nucleotide similarity values of these viruses are with the attenuated isolates CEF94 and D78 (above 99%).

**Distinct infectious bursal disease viruses.** Seven Uruguayan (UY-42/07, UY-04/09, UY-06/10, UY-07/10, UY-171101, UY-421101 and UY-221201) and two Argentine (AR-C1 and AR-C3) viruses have a singular nucleotide and amino acidic composition. The VP2 hypervariable region consistently shows the 222S marker, and the signature 272T, 289P, 290I and 296F that in other viruses corresponds to 272V, 289L, 290M/L and 296I. These dIBDVs have similarity values above 99% with particular viruses from Argentina (e.g. 05A25, P30903), Brazil (e.g. B8), Colombia (01C5), Canada (e.g. 03–42857, 05-32115-B9), Puerto Rico (586), Hungary (e.g. P10, P3), Poland (78/GSz, 80GA), Russia (IBDVRF-8/96) and South Korea (K310), which have the same 272T, 289P, 290I and 296F signature.

Phylogenetic analysis using Argentine, Uruguayan and global IBDVs, including common reference strains and IBDVs from different years and regions, was performed to explore IBDV relationships based on the hvVP2 coding region. The phylogenetic analysis showed five well-supported clades corresponding to cIBDVs, caIBDVs, vaIBDVs, vvIBDVs and dIBDVs (Figure 2). The Argentine and Uruguayan IBDVs fall within the corresponding clusters according with the initial classification based on sequence comparison and amino acid markers.

Additional approaches were implemented to further analyse IBDV clustering. First, DAPC was carried out to infer the clustering pattern of IBDVs. Five clearly distinguishable groups were obtained and corresponded to the same clades identified in the phylogenetic cladogram (Figure 3A). When considering just the first discriminant function retained during the principal components analysis (which explained 70% of the genetic variance in the data-set), IBDV groups are still distinguishable (Figure 3B). Second, the net between groups mean distances were calculated, showing that vvIBDVs (median = 0.17) and dIBDVs (median = 0.14) are the most divergent groups (Figure 3C).

To gain insight into the variability of dIBDVs, we sequenced a region of the *vp1* gene in some of these viruses. VP1 has the non-vvIBDV markers 146E, 147G and 242D and a unique F243P change (Figure 4). The phylogeny recovers the vv lineage and a non-vvIBDV clade that includes the dIBDVs (Figure 5). The only available VP1 sequences for members of the dIBDV lineage were the Uruguayan IBDVs and the Poland isolates 78GsZ and 80GA (Figures 4 and 5). These dIBDVs constitute a closely related monophyletic group in the VP1 phylogeny (Figure 5).

## Discussion

Three major events describe the evolutionary history of the infectious bursal disease; the detection of cIBDVs in the early 1960s, and the almost simultaneous emergence of va and vv strains about 25 years later. Genetic analyses revealed that these IBDV strains have nucleotide and amino acid signatures and constitute well-supported independent clades in phylogenetic trees (Boot *et al.*, 2000; van den Berg, 2000; Le Nouën *et al.*, 2005; Hon *et al.*, 2006). Besides the three lineages corresponding to c, va and vv strains, phylogenetic trees also resolve an independent lineage for the ca strains that comprise vaccine strains maintained in the laboratory and vaccine-like virus collected from the field (Kim *et al.*, 2010).

In order to map IBDV diversity in the southern poultry industry of South America, we characterized viruses from Argentina and Uruguay. Genetic analysis of the hvVP2 coding region revealed a great genetic diversity of the IBDVs circulating in the surveyed poultry production. With exception of the vaIBDVs, the remaining strains (c, vv and ca) are present in Argentina and Uruguay. Strain classification was unambiguously performed by the presence of molecular markers and sequence similarity with reference or previously classified IBDVs, and by phylogenetic analysis that clustered the viruses into well-defined lineages (Figures 1 and 2).

Remarkably, the majority of the characterized viruses (9 out of 15) have particular amino acid residues and grouped together in a well-supported clade separated from the pre-existing IBDV strains (Figures 1 and 2). Accordingly, the genetic analysis cannot accurately assign this group of viruses, here denoted as dIBDVs, to the typical c, ca, va and vv strains. These Uruguayan and Argentine dIBDVs are included in an independent evolutionary lineage with previously reported viruses from other countries in America (Jackwood & Sommer, 1997; Ikuta *et al.*, 2001; Banda *et al.*, 2003; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007), Europe (Domanska *et al.*, 2004) and Asia (Kwon *et al.*, 2000) (Figure 2). The hvVP2

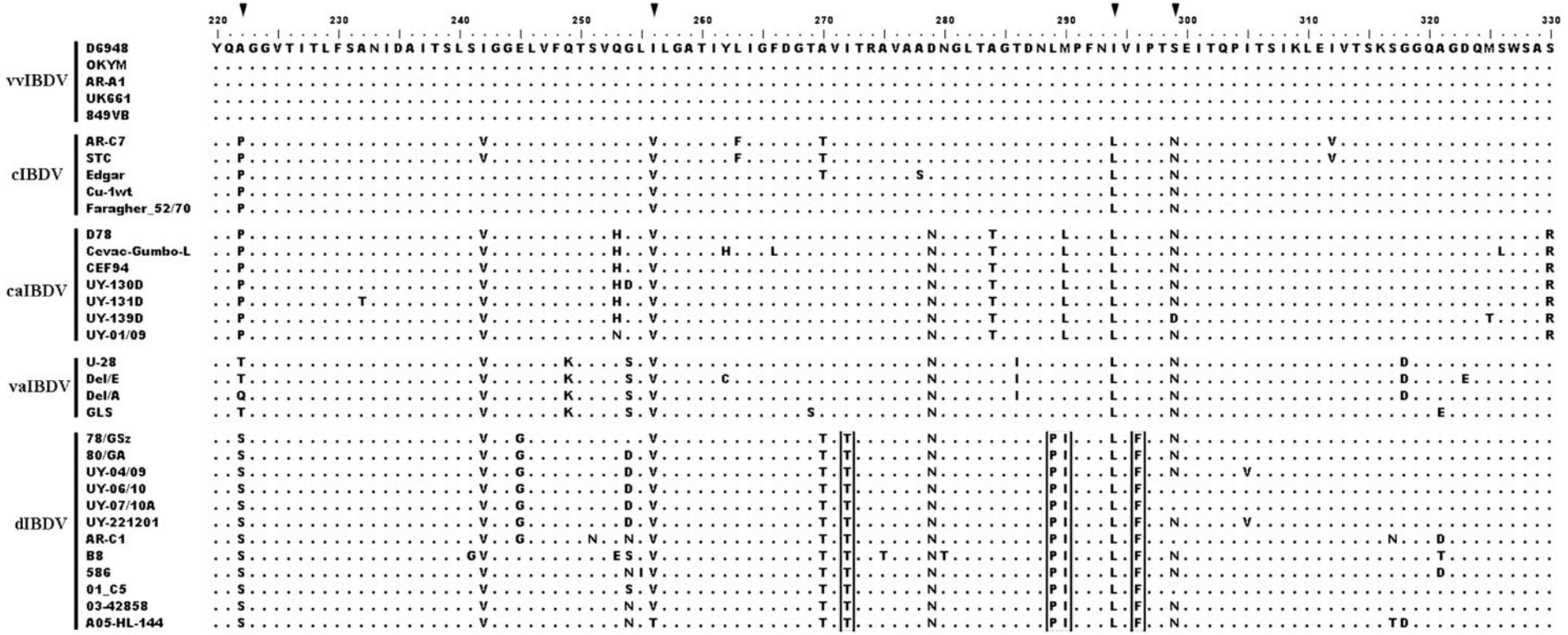


**Figure 1.** *Amino acid differences along the coding sequences of the hvVP2 region of very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs, and representatives of the distinct IBDVs (dIBDVs). Dots indicate position where the sequences are identical to the segment A of the hypervirulent D6948 strain (AF240686). Arrowheads indicate common amino acid markers used to distinguish hypervirulent from non-hypervirulent strains; the dIBDV group shares markers with both groups. The hvVP2 sequences of the dIBDV lineage have a highly conserved molecular signature: 272T, 289P, 290I and 296F (indicated by boxes). This signature may be considered a diagnostic character for the dIBDV lineage.*

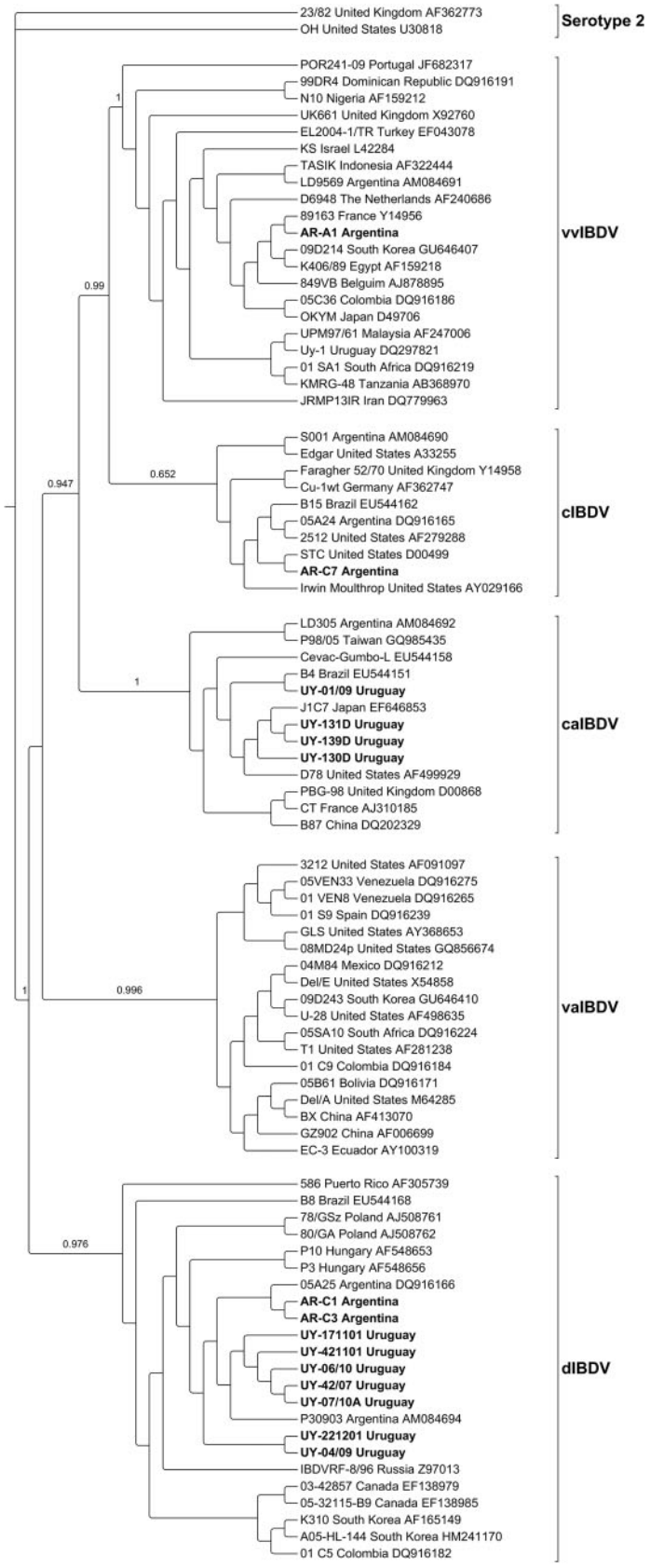


**Figure 2.** *Cladogram inferred by using the maximum-likelihood method with TIM2ef+I+G substitution model. Phylogenetic reconstruction was carried out using a 374-bp fragment (nucleotide positions 795–1168) that codes for the hvVP2 region. Uruguayan and Argentine IBDVs characterized in this study are indicated in bold. Name, origin and accession number of each sequence are denoted. Mapping uncertainties for interesting internal nodes are shown as approximate likelihood ratio test values. Besides serotype 2 strains, five well-supported clades are distinguishable: very virulent (vv), classic (c), classic attenuated (ca), variant (va) and distinct viruses (dIBDVs). Nine out of the 15 characterized IBDVs were grouped into the dIBDV clade. This lineage comprises viruses from America, Europe and Asia.*

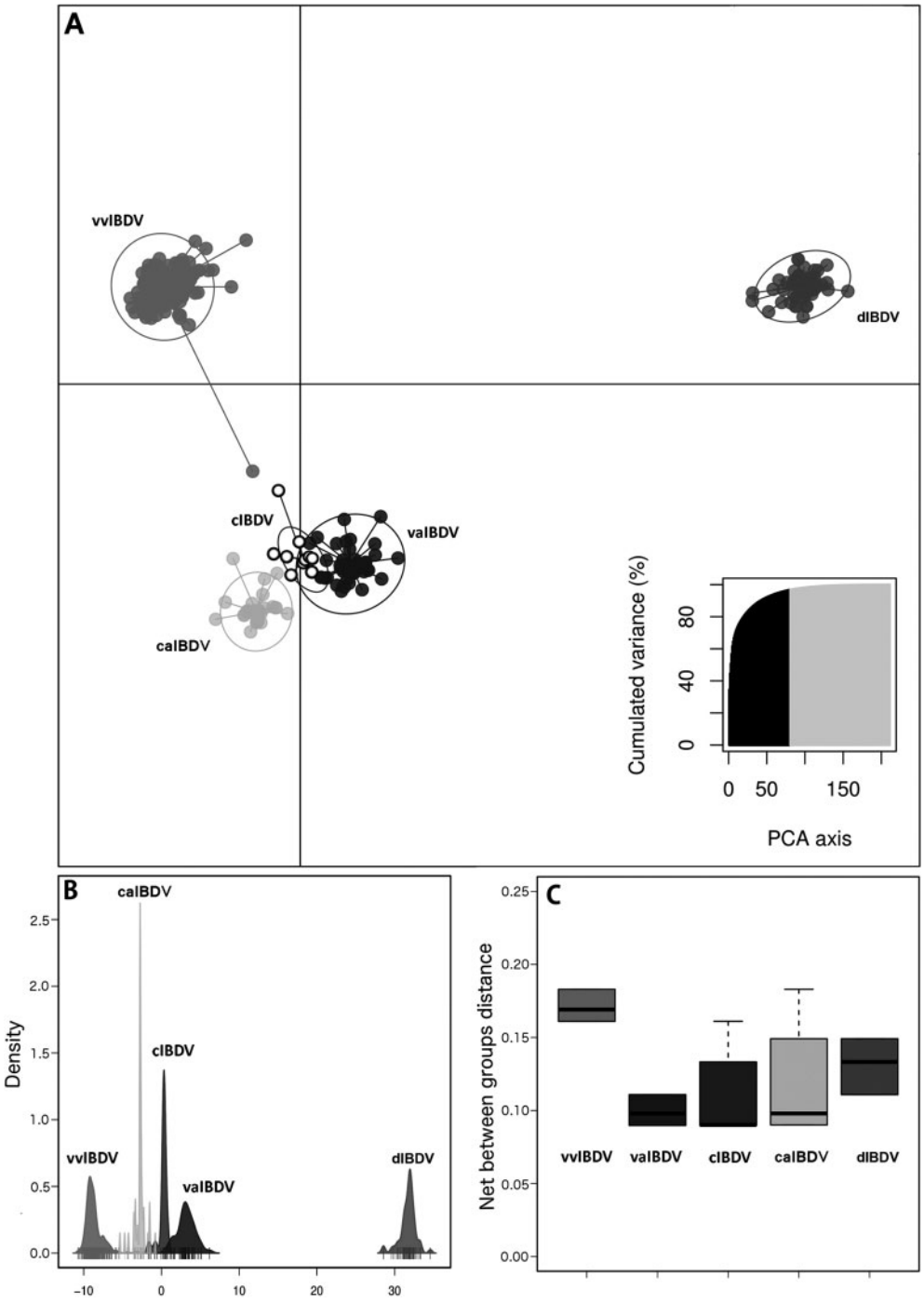


**Figure 3.** *(A) Discriminant analysis of principal components (DAPC). The data-set included the VP2 nucleotide sequences used in the phylogenetic analysis and additional representatives of the different IBDV strains (N=423). Groups are shown by different grey shades and inertia ellipses, while dots represent individual viruses. Five clusters are distinguished and correspond to the four traditional very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs and to the distinct IBDV group (dIBDV) described in the present study. The dIBDV and vvIBDV strains have the greatest between group variance, indicating that they are the most genetically divergent groups. (B) First simple discrimination function retained during the principal component analysis. This analysis explains 70% of the genetic variance in the data-set and correctly discriminates the five IBDV clusters. (C) Net between-groups mean distances. The vvIBDV (mean = 0.17) and dIBDV strains (mean = 0.14) show the highest divergence.*

sequence of the dIBDVs has a molecular signature (272T, 289P, 290I and 296F) that is unique, conserved and here suggested as a diagnostic character for the lineage (Figure 1). The dIBDVs share relevant amino acids markers (222S, 256V, 294L and 299N/S) with both vvIBDV and non-vvIBDV strains. The 222S residue was already reported in the classic Lukert strain, in few vvIBDVs, and in a Belgian isolate that was considered a European variant with a different antigenic profile produced by the P222S change (Rudd *et al.*, 2002; Jackwood & Sommer-Wagner, 2007; Letzel *et al.*, 2007). The 256V and 294L residues are characteristic of the non-vvIBDVs, while the dIBDVs have a 299N, like most non-vvIBDVs, or 299S, as most vvIBDVs. Notably, the Polish 78/GSz and 80/GA and Hungarian P3 and P10 isolates collected from outbreaks during 1975–1981 grouped within the dIBDVs clade in the VP2 phylogeny. These viruses have a singular antigenic profile and belong to a group of six isolates which were suggested to have

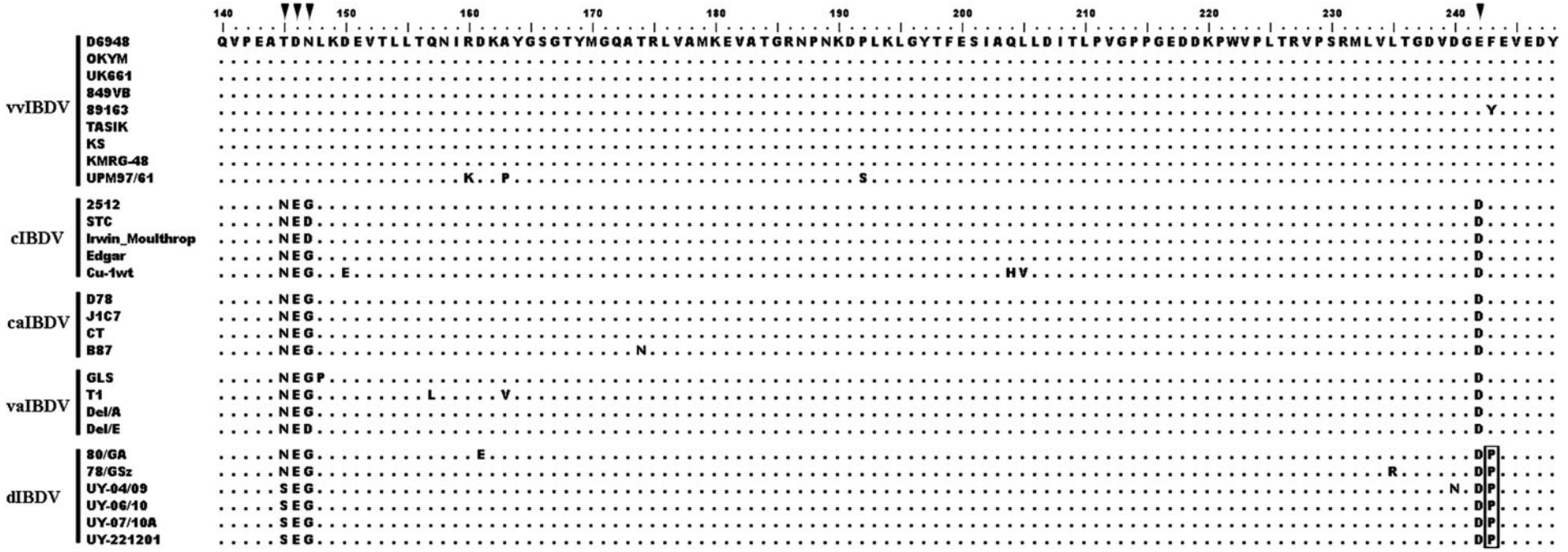


**Figure 4.** *Amino acid differences along partial VP1 coding region of very virulent (vv), classic (c), classic attenuated (ca) and variant (va) IBDVs, and representatives of the distinct IBDV group (dIBDV). Dots indicate position where the sequences are identical to the segment B of the hypervirulent D6948 strain (AF240687). Arrowheads indicate common amino acid markers used to distinguish hypervirulent from non-hypervirulent strains; the dIBDV group shares markers with the non-hypervirulent group. The VP1 sequences of the dIBDV lineage have a highly conserved proline residue (indicated by a box). This residue may be considered a diagnostic character for the dIBDV lineage in the VP1 gene.*

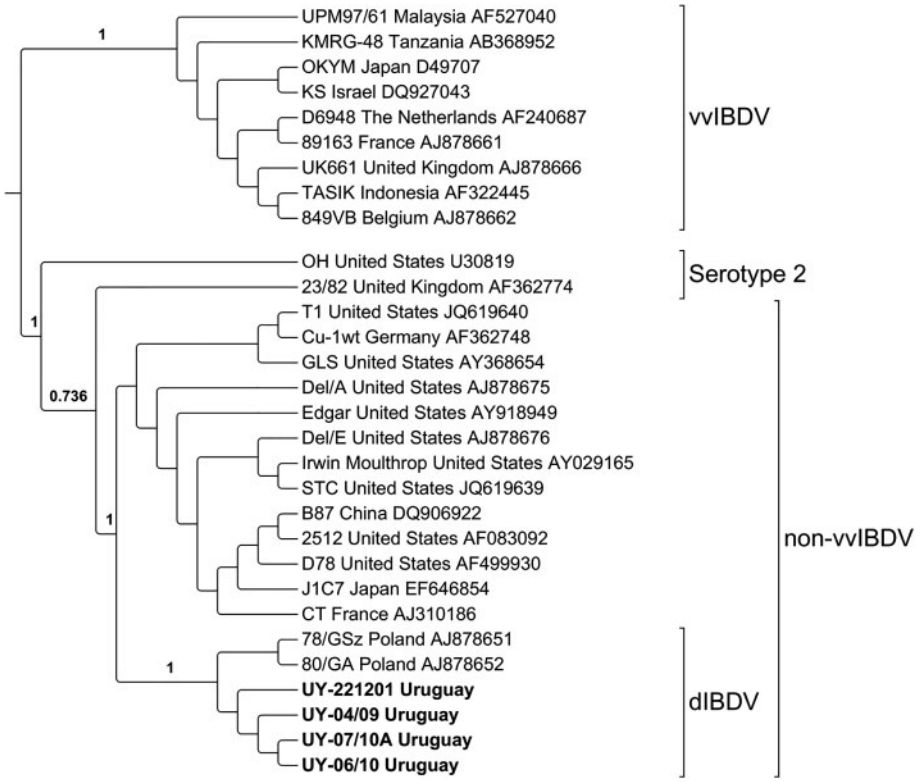


**Figure 5.** *Cladogram inferred by using the maximum-likelihood method with K80+I+G substitution model. Phylogenetic reconstruction was carried out using a 594-bp fragment (nucleotide positions 322–855 of the vp1 gene). Uruguayan and Argentine IBDVs characterized in this study are indicated in bold. Name, origin and accession number of each sequence are denoted. Mapping uncertainties for interesting internal nodes are shown as approximate likelihood ratio test values. The cladogram displays two major clades conformed by the vvIBDVs and non-vvIBDVs: classic attenuated, variant, classic IBDVs and the dIBDVs here defined.*

coexisted and being later displaced by "F52/70-like" viruses before the vvIBDV emergence in Europe (Domanska *et al.*, 2004). Rather than being extinct, our findings suggest that this lineage has been circulating worldwide for decades and is currently coexisting with traditional strains, reaching high prevalence in countries such as Argentina and Uruguay.

We explore the independence of this distinct lineage using DAPC, a novel-specific multivariate method that identifies and describes clusters of genetically related entities maximizing the separation between groups and minimizing within-group variation (Jombart *et al.*, 2010). This method has been extensively applied in viruses, bacteria, eukaryotic unicellular parasites and metazoans to reveal population structuring using different genetic markers (Jombart *et al.*, 2010; Ramírez *et al.*, 2012; Ogawa & Vallender, 2014; Vernière *et al.*, 2014). The analysis resolves five clusters corresponding to the four traditional IBDV strains plus the group of dIBDVs (Figure 3). The dIBDVs and vvIBDVs have the highest between-group variance (Figure 3A) and genetic distance (Figure 3C), indicating that both are the most genetically divergent groups of IBDVs. These results reinforce those obtained by the sequence and phylogenetic analyses and add evidence about the separation of the dIBDV lineage.

The divergence of the dIBDVs involves both genomic segments since these viruses also have unique markers (243P) and a particular phylogenetic clustering when using segment B (*vp1* gene) sequences (Figures 4 and 5). The dIBDVs have the markers 146E, 147G and 242D characteristic of the non-vv viruses (Hernández *et al.*, 2006). Non-vvIBDVs are usually associated with less pathogenicity than vvIBDVs (van den Berg, 2000), a feature that is consistent with the absence of notorious clinical manifestations of the Uruguayan and Argentine dIBDVs. Coincidentally, subclinical manifestations were reported for several of the viruses clustered within the dIBDV lineage, suggesting that low pathogenicity is a common feature in this group of viruses (Ikuta *et al.*, 2001; Smiley & Jackwood, 2001).

The isolates 78GsZ and 80GA from Poland appear closely related with the Uruguayan dIBDVs in the VP1 cladogram (Figure 5), despite being collected nearly four decades ago (Domanska *et al.*, 2004). This result reinforces the similarity between the Uruguayan dIBDVs and the Polish viruses, which had unique genetic and antigenic changes and low-pathogenicity.

Together, our findings reveal the existence of an IBDV evolutionary lineage highly divergent from the traditional strains that are defined by a unique amino acid signature. This lineage was detected with a high frequency co-circulating with typical strains in Argentina and Uruguay, but it spread in countries from other continents. Its wide geographic distribution, the existence of old European strains and the high divergence revealed by the DAPC analysis suggest that this lineage is ancient and has remained overlooked for a long time. Their sporadic detection and the lack of specific comparative studies (e.g. DAPC and up-to-date phylogenetic methods) would explain why these viruses were considered extinct or atypical isolates restricted to spatially confined regions, instead of being defined as a discrete evolutionary lineage with an ancient origin. It is possible that these dIBDVs were easily ignored in routine surveillances because of the lack of well known clinical signs.

The circulation of this divergent and widespread IBDV strain represents a very important finding that updates and

helps the understanding of the current epidemiology of the prevalent infectious bursal disease.

## Acknowledgement

We thank Granjas Hepa Ltda. and the Asociación Colombiana de Médicos Veterinarios y Zootecnistas Especialistas en Avicultura for collaboration.

## Funding

This work was supported by the Instituto Nacional de Investigación Agropecuaria (Fondo de Promoción de Tecnología Agropecuaria) under a grant [no. 319], and the Agencia Nacional de Investigación e Innovación under a grant [no. FMV-7073].

## References

Azad, A.A., Jagadish, M.N., Brown, M.A. & Hudson, P.J. (1987). Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. *Virology*, 161, 145–152.

Banda, A. & Villegas, P. (2004). Genetic characterization of very virulent infectious bursal disease viruses from Latin America. *Avian Diseases*, 48, 9–18.

Banda, A., Villegas, P. & El-Attrache, J. (2003). Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. *Avian Diseases*, 47, 87–95.

Bayliss, C.D., Spies, U., Shaw, K., Peters, R.W., Papageorgiou, A., Müller, H. & Boursnell, M.E. (1990). A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *The Journal of General Virology*, 71, 1303–1312.

Boot, H.J., ter Huurne, A.A., Hoekman, A.J., Peeters, B.P. & Gielkens, A.L. (2000). Rescue of very virulent and mosaic infectious bursal disease virus from cloned cDNA: VP2 is not the sole determinant of the very virulent phenotype. *Journal of Virology*, 74, 6701–6711.

Cao, Y.C., Yeung, W.S., Law, M., Bi, Y.Z., Leung, F.C. & Lim, B.L. (1998). Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. *Avian Diseases*, 42, 340–351.

Chettle, N., Stuart, J.C. & Wyeth, P.J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. *The Veterinary Record*, 125, 271–272.

Cosgrove, A.S. (1962). An apparently new disease of chickens: avian nephrosis. *Avian Diseases*, 6, 385.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology*, 76, 2393–2402.

Domanska, K., Mato, T., Rivallan, G., Smietanka, K., Minta, Z., de Boisseson, C., Toquin, D., Lomniczi, B., Palya, V. & Eterradossi, N. (2004). Antigenic and genetic diversity of early European isolates of infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of infectious bursal disease virus revisited? *Archives of Virology*, 149, 465–480.

Edgar, S.A. & Cho, Y. (1976). The epizootiology of infectious bursal disease and prevention of it by immunization. *Developments in Biological Standardization*, 33, 349–356.

Escaffre, O., Le Nouën, C., Amelot, M., Ambroggio, X., Ogden, K.M., Guionie, O., Toquin, D., Müller, H., Islam, M.R. & Eterradossi, N. (2013). Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. *Journal of Virology*, 87, 80–93.

Fahey, K.J., Chapman, A.J., Macreadie, I.G., Vaughan, P.R., McKern, N.M., Skicko, J.I., Ward, C.W. & Azad, A.A. (1991). A recombinant subunit vaccine that protects progeny chickens from infectious bursal disease. *Avian Pathology*, 20, 447–460.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704.

Hernández, M., Banda, A., Hernández, D., Panzera, F. & Pérez, R. (2006). Detection of very virulent strains of infectious bursal disease virus (vvIBDV) in commercial broilers from Uruguay. *Avian Diseases*, 50, 624–631.

Hon, C.C., Lam, T.Y., Drummond, A., Rambaut, A., Lee, Y.F., Yip, C.W., Zeng, F., Lam, P.Y., Ng, P.T.W. & Leung, F.C.C. (2006). Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. *Journal of Virology*, 80, 8503–8509.

Ikuta, N., El-Attrache, J., Villegas, P., Garcia, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. & Marques, E.K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Diseases*, 45, 297–306.

Islam, M.A.T., Le, T.H. & Rahman, M.M. (2012). Molecular characterization of two Bangladeshi infectious bursal disease virus isolates using the hypervariable sequence of VP2 as a genetic marker. *Journal of Veterinary Science*, 13, 405–412.

Ismail, N.M., Saif, Y.M., Wigle, W.L., Havenstein, G.B. & Jackson, C. (1990). Infectious bursal disease virus variant from commercial Leghorn pullets. *Avian Diseases*, 34, 141–145.

Jackwood, D.J., Saif, Y.M. & Moorhead, P.D. (1985). Immunogenicity and antigenicity of infectious bursal disease virus serotypes I and II in chickens. *Avian Diseases*, 29, 1184–1194.

Jackwood, D.J. & Sommer, S.E. (1997). Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses. *Avian Diseases*, 41, 627–637.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal disease viruses from four continents. *Virology*, 365, 369–375.

Jombart, T., Devillard, S. & Balloux, F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, 11, 94.

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30, 3059–3066.

Kim, H.R., Kwon, Y.K., Bae, Y.C., Oem, J.K. & Lee, O.S. (2010). Genetic characteristics of virion protein 2 genes of infectious bursal disease viruses isolated from commercial chickens with clinical disease in South Korea. *Poultry Science*, 89, 1642–1646.

Kwon, H.M., Kim, D.K., Hahn, T.W., Han, J.H. & Jackwood, D.J. (2000). Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. *Avian Diseases*, 44, 691–696.

Le Nouën, C., Rivallan, G., Toquin, D., Darlu, P., Morin, Y., Beven, V., de Boisseson, C., Cazaban, C., Comte, S., Gardin, Y. & Eterradossi, N. (2006). Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment-B-reassorted isolate. *The Journal of General Virology*, 87, 209–216.

Le Nouën, C., Rivallan, G., Toquin, D. & Eterradossi, N. (2005). Significance of the genetic relationships deduced from partial nucleotide sequencing of infectious bursal disease virus genome segments A or B. *Archives of Virology*, 150, 313–325.

Lejal, N., Costa, B.D., Huet, J.C., Delmas, B. & Da Costa, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology*, 81, 983–992.

Letzel, T., Coulibaly, F., Rey, F.A., Delmas, B., Jagt, E., van Loon, A.A.M. W. & Mundt, E. (2007). Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *Journal of Virology*, 81, 12827–12835.

Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodríguez, J.F. & Castón, J.R. (2009). Infectious bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *Journal of Molecular Biology*, 386, 891–901.

Martin, A.M., Fallacara, F., Barbieri, I., Tosi, G., Rivallan, G., Eterradossi, N., Ceruti, R. & Cordioli, P. (2007). Genetic and antigenic characterization of infectious bursal disease viruses isolated in Italy during the period 2002–2005. *Avian Diseases*, 51, 863–872.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. & Allan, G.M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. *Avian Pathology*, 9, 395–404.

Müller, H., Scholtissek, C. & Becht, H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. *Journal of Virology*, 31, 584–589.

Ogawa, L.M. & Vallender, E.J. (2014). Genetic substructure in cynomolgus macaques (*Macaca fascicularis*) on the island of Mauritius. *BMC Genomics*, 15, 748. London: BioMed Central.

Ojkic, D., Martin, E., Swinton, J., Binnington, B. & Brash, M. (2007). Genotyping of Canadian field strains of infectious bursal disease virus. *Avian Pathology*, 36, 427–433.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, 25, 1253–1256.

R Development Core Team. (2013). R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/

Ramírez, J.D., Guhl, F., Messenger, L.A., Lewis, M.D., Montilla, M., Cucunuba, Z., Miles, M.A. & Llewellyn, M.S. (2012). Contemporary cryptic sexuality in *Trypanosoma cruzi*. *Molecular Ecology*, 21, 4216–4226.

Remorini, P., Calderón, M.G., Aguirre, S., Periolo, O., La Torre, J. & Mattion, N. (2006). Characterization of infectious bursal disease viruses from Argentina. *Avian Diseases*, 50, 245–251.

Rosales, A.G., Villegas, P., Lukert, P.D., Fletcher, O.J. & Brown, J. (1989). Immunosuppressive potential and pathogenicity of a recent isolate of infectious bursal disease virus in commercial broiler chickens. *Avian Diseases*, 33, 724–728.

Rudd, M., Heine, H., Sapats, S., Parede, L. & Ignjatovic, J. (2002). Characterisation of an Indonesian very virulent strain of infectious bursal disease virus. *Archives of Virology*, 147, 1303–1322.

Sánchez, A.B. & Rodriguez, J.F. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology*, 262, 190–199.

Sharma, J.M., Dohms, J.E. & Metz, A.L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. *Avian Diseases*, 33, 112–124.

Smiley, J.R. & Jackwood, D.J. (2001). Genetic stability of the VP2 hypervariable region of four infectious bursal disease virus isolates after serial passage in specific-pathogen-free chicken embryos. *Avian Diseases*, 45, 1–8.

Snedeker, C., Wills, F.K. & Moulthrop, I.M. (1967). Some studies on the infectious bursal agent. *Avian Diseases*, 11, 519–528.

Spies, U., Müller, H. & Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Research*, 8, 127–140.

Stöver, B.C. & Müller, K.F. (2010). TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11, 7.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Tomás, G., Hernández, M., Marandino, A., Panzera, Y., Maya, L., Hernández, D., Pereda, A., Banda, A., Villegas, P., Aguirre, S. & Pérez, R. (2012). Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. *Journal of Virological Methods*, 185, 101–107.

Vakharia, V.N., He, J., Ahamed, B. & Snyder, D.B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. *Virus Research*, 31, 265–273.

Van den Berg, T.P. (2000). Acute infectious bursal disease in poultry: a review. *Avian Pathology*, 29, 175–194.

Van den Berg, T.P., Gonze, M. & Meulemans, G. (1991). Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. *Avian Pathology*, 20, 133–143.

Van den Berg, T.P., Gonze, M., Morales, D. & Meulemans, G. (1996). Acute infectious bursal disease in poultry: immunological and molecular basis of antigenicity of a highly virulent strain. *Avian Pathology*, 25, 751–768.

Vernière, C., Bui Thi Ngoc, L., Jarne, P., Ravigné, V., Guérin, F., Gagnevin, L., Le Mai, N., Chau, N.M. & Pruvost, O. (2014). Highly polymorphic markers reveal the establishment of an invasive lineage of the citrus bacterial pathogen *Xanthomonas citri* pv. *citri* in its area of origin. *Environmental Microbiology*, 16, 2226–2237.

Wu, C.C., Rubinelli, P. & Lin, T.L. (2007). Molecular detection and differentiation of infectious bursal disease virus. *Avian Diseases*, 51, 515–526.

Wu, Y., Hong, L., Ye, J., Huang, Z. & Zhou, J. (2009). The VP5 protein of infectious bursal disease virus promotes virion release from infected cells and is not involved in cell death. *Archives of Virology*, 154, 1873–1882.

Xia, R.X., Wang, H.Y., Huang, G.M. & Zhang, M.F. (2008). Sequence and phylogenetic analysis of a Chinese very virulent infectious bursal disease virus. *Archives of Virology*, 153, 1725–1729.

Yamaguchi, T., Kasanga, C.J., Terasaki, K., Maw, M.T., Ohya, K. & Fukushi, H. (2007). Nucleotide sequence analysis of VP2 hypervariable domain of infectious bursal disease virus detected in Japan from 1993 to 2004. *The Journal of Veterinary Medical Science*, 69, 733–738.

genomeA
Journals.ASM.org

# Genome Sequence of a Distinct Infectious Bursal Disease Virus

**Gonzalo Tomás, Martín Hernández, Ana Marandino, Diego Hernández, Claudia Techera, Sofía Grecco, Yanina Panzera, Ruben Pérez**

Facultad de Ciencias, Sección Genética Evolutiva, Instituto de Biología, Universidad de la República, Montevideo, Uruguay

**Infectious bursal disease virus is a relevant avian pathogen that affects poultry production. Here, we report the full-length coding sequence of the Uruguayan strain dIBDV/UY/2014/2202, isolated from a commercial broiler flock. The strain belongs to the distinct IBDV lineage that is widely distributed in South America.**

**Received** 3 August 2015 **Accepted** 17 August 2015 **Published** 1 October 2015

**Citation** Tomás G, Hernández M, Marandino A, Hernández D, Techera C, Grecco S, Panzera Y, Pérez R. 2015. Genome sequence of a distinct infectious bursal disease virus. Genome Announc 3(5):e01061-15. doi:10.1128/genomeA.01061-15.



Address correspondence to Ruben Pérez, rperez@fcien.edu.uy.

Infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus* within the family *Birnaviridae*. This nonenveloped icosahedral virus has a double-stranded RNA genome consisting of two segments named A and B (1).

IBDV is a highly contagious avian pathogen affecting commercial poultry production worldwide (2). All pathogenic IBDVs (serotype 1) can be divided into classic, variant, and very virulent strains according to antigenic and pathogenic criteria (3). Phylogenetic analyses have consistently recovered the clades or lineages corresponding to these strains, including a clade composed of vaccine-like strains and a recently described distinct global lineage (4). This distinct lineage (dIBDV) is widely distributed in South America (4, 5).

The IBDV strain was collected in 2014 from a 27-day-old commercial broiler flock suffering from respiratory problems and increased mortality. Viral RNA was isolated from bursae tissue using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using random hexamers (Thermo Scientific). The IBDV was diagnosed as a non-very virulent virus by real-time PCR (6). The complete genome sequence of this isolate was obtained by reverse transcription PCR, using overlapped consensus primers and direct sequencing. Purified products were sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). Sequences were compiled and edited using the SeqMan program (Lasergene). Multisequence alignments were performed with MEGA5 (7), and phylogenetic trees were constructed using PhyML.

The coding region of segment A (3,073 nucleoides) contains two open reading frames that encode VP5 (145 aa) and a VP2-VP4-VP3 polyprotein (1,012 aa); segment B encodes VP1 (879 aa), the viral RNA polymerase.

The phylogenetic analysis of the VP2 hypervariable region indicates that the virus belongs to the dIBDV lineage. This was confirmed by the presence of a unique and conserved molecular signature (272T, 289P, 290I, and 296F) that is a diagnostic character for the classification of dIBDVs (4). Segment B also associates with strains of the dIBDV lineage and shows the 243P diagnostic marker. Consequently, the strain was denoted as dIBDV/UY/2014/2202. This is the first full-length sequence of the coding genome of a strain belonging to the dIBDV lineage.

Comparative analysis of the complete segment A shows higher nucleotide similarity (96%) with classical strains (e.g., Edgar and HPR-2). In the case of VP1 (segment B), the highest similarity (96.4%) was found with strains that are not very virulent (e.g., JD1 and Irwin Moulthrop).

The comparison of the VP2 hypervariable region of dIBDV/UY/2014/2202 with most vaccine strains commonly used in South America showed an amino acid similarity ranging from 88.9 to 92.1%; D78 (Nobilis D78) and Winterfield 2512 (Cevac IBD L) were the most similar strains.

A comprehensive study of more genomic sequences of these dIBDVs is needed to understand the virus's epidemiology and to contribute to the effective control of IBDV infection.

**Nucleotide sequence accession numbers.** The full-length coding sequence of strain dIBDV/UY/2014/2202 has been deposited in GenBank under the accession numbers KT336459 (segment A) and KT336458 (segment B).

## ACKNOWLEDGMENT

This work was supported by the Instituto Nacional de Investigación Agropecuaria (Fondo de Promoción de Tecnología Agropecuaria) under grant 319 and by the Comisión Sectorial de Investigación Científica (BECA_INI_2013 ID_79).

## REFERENCES

1. **Müller H, Scholtissek C, Becht H, Muller H.** 1979. The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. J Virol **31:**584–589.
2. **Eterradossi N, Saif YM.** 2008. Infectious bursal disease, p 185–208. *In* Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (ed), Diseases of poultry, 12th ed. Blackwell Publishing, Ames, IA.
3. **Van den Berg TP, Morales D, Eterradossi N, Rivallan G, Toquin D, Raue R, Zierenberg K, Zhang MF, Zhu YP, Wang CQ, Zheng HJ, Wang X, Chen GC, Lim BL, Müller H.** 2004. Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. Avian Pathol **33:**470–476. http://dx.doi.org/10.1080/03079450400003650.
4. **Hernández M, Tomás G, Marandino A, Iraola G, Maya L, Mattion N, Hernández D, Villegas P, Banda A, Panzera Y, Pérez R.** 2015. Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. Avian Pathol **44:**212–221. http://dx.doi.org/10.1080/03079457.2015.1025696.
5. **Vera F, Craig MI, Olivera V, Rojas F, König G, Pereda A, Vagnozzi A.**

2015. Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. Arch Virol **160:** 1909–1921. http://dx.doi.org/10.1007/s00705-015-2449-4.

6. **Tomás G, Hernández M, Marandino A, Panzera Y, Maya L, Hernández D, Pereda A, Banda A, Villegas P, Aguirre S, Pérez R**. 2012. Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. J Virol Methods **185:**101–107. http://dx.doi.org/10.1016/j.jviromet.2012.06.012.
7. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S**. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.

AVIAN PATHOLOGY, 2017
VOL. 46, NO. 2, 150–156
http://dx.doi.org/10.1080/03079457.2016.1228827

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

# Development of an RT-qPCR assay for the specific detection of a distinct genetic lineage of the infectious bursal disease virus

Gonzalo Tomás[a], Martín Hernández[a], Ana Marandino[a], Claudia Techera[a], Sofia Grecco[a], Diego Hernández[a], Alejandro Banda[b], Yanina Panzera[a] and Ruben Pérez[a]

[a]Sección Genética Evolutiva, Departamento de Biología Animal, Instituto de Biología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay; [b]Poultry Research and Diagnostic Laboratory, College of Veterinary Medicine, Mississippi State University, Pearl, MS, USA

**ABSTRACT**

The infectious bursal disease virus (IBDV) is a major health threat to the world's poultry industry despite intensive controls including proper biosafety practices and vaccination. IBDV (*Avibirnavirus, Birnaviridae*) is a non-enveloped virus with a bisegmented double-stranded RNA genome. The virus is traditionally classified into classic, variant and very virulent strains, each with different epidemiological relevance and clinical implications. Recently, a novel worldwide spread genetic lineage was described and denoted as distinct (d) IBDV. Here, we report the development and validation of a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay for the specific detection of dIBDVs in the global poultry industry. The assay employs a TaqMan-MGB probe that hybridizes with a unique molecular signature of dIBDV. The assay successfully detected all the assessed strains belonging to the dIBDV genetic lineage, showing high specificity and absence of cross-reactivity with non-dIBDVs, IBDV-negative samples and other common avian viruses. Using serial dilutions of *in vitro*-transcribed RNA we obtained acceptable PCR efficiencies and determination coefficients, and relatively small intra- and inter-assay variability. The assay demonstrated a wide dynamic range between $10^3$ and $10^8$ RNA copies/reaction. This rapid, specific and quantitative assay is expected to improve IBDV surveillance and control worldwide and to increase our understanding of the molecular epidemiology of this economically detrimental poultry pathogen.

**ARTICLE HISTORY**
Received 9 May 2016
Accepted 21 August 2016

**KEYWORDS**
IBDV; RT-qPCR; TaqMan-MGB probe; distinct IBDV; IBD

## Introduction

Infectious bursal or Gumboro disease is a highly contagious viral affection that causes major economic losses in the global poultry industry. The aetiologic agent is the infectious bursal disease virus (IBDV) belonging to the genus *Avibirnavirus* within the family *Birnaviridae*. The virus replicates in the B lymphocytes of the bursa of Fabricius and affects the immune system of immature chickens, leading to an increased susceptibility to other infectious diseases and a poor antibody response to vaccines (Rosenberger & Gelb, 1978).

IBDV is a non-enveloped icosahedral virus with a bisegmented, double-stranded RNA genome (Müller *et al.*, 1979). Segment A (3.3 kpb) has two partially overlapped open reading frames (ORFs). ORF A-1 encodes a precursor polyprotein that is autocatalytically cleaved into the immature outer capsid protein pVP2, the viral protease VP4 and the ribonucleoprotein VP3 (Sánchez *et al.*, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002; Luque *et al.*, 2009). The pVP2 protein is further processed to yield the mature VP2, the major host-protective antigen of IBDV (Fahey *et al.*, 1991). ORF A-2 encodes VP5, a non-structural protein involved in virion release from infected cells (Wu *et al.*, 2009). Genomic segment B (2.9 kpb) encodes the RNA-dependent RNA polymerase VP1 (Spies *et al.*, 1987).

There are two IBDV serotypes (1 and 2), but only serotype 1 comprises pathogenic viruses (Ismail *et al.*, 1988). This serotype was traditionally divided into classic (c), variant (va) and very virulent (vv) strains using antigenic and pathogenic criteria. Classic strains can be further classified into classic virulent (cv) and attenuated vaccine strains, usually referred to as "classic attenuated" (ca) IBDVs.

Recently, we described the existence of a worldwide spread genetic lineage denoted as distinct (d) IBDV (Hernández *et al.*, 2015). This lineage is resolved in a well-supported clade and has a unique four-amino-acid signature (T272, P289, I290, F296). Most isolates that are now classified as dIBDV were initially considered atypical classic or variant strains that harboured unique nucleotide and amino acid changes as a consequence of local differentiation (Kwon *et al.*, 2000; Ikuta *et al.*, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007). Some dIBDV isolates have exhibited mild clinical signs and antigenic differences (Ikuta

**CONTACT** Ruben Pérez rperez@fcien.edu.uy
Supplemental data for this article can be accessed 10.1080/03079457.2016.1228827

**Table 1.** Description of primers and probe used in this study.

| Primer/probe name | Sequence 5′→ 3′ | Polarity | Position[a] | Amplicon size |
|---|---|---|---|---|
| F964rt | AAACAATGGGCTRACGGC | + | 964–981 | 72 |
| R1035rt | GTTATCTCGYTGGTCGGRAA | – | 1035–1016 | |
| P1011rt | NED-AGRTTGAATGGAAYAGGA-MGB-NFQ | – | 1011–994 | |

[a]Sequences numbering according to segment A of the vvIBDV strain D6948 (AF240686).

*et al.*, 2001; Domanska *et al.*, 2004; Vera *et al.*, 2015) but further phenotypic studies are needed to understand the epidemiological and sanitary relevance of this lineage that contains part of the genetic variability of the virus (Hernández *et al.*, 2015).

Global surveillance and research programmes require reliable assays for the diagnosis of IBDV variants to understand virus spreading and evolution, and to provide strain-specific treatments (Van den Berg *et al.*, 2000). Strain classification can be performed by the phylogenetic analysis of the VP2 hypervariable region (hvVP2), which recovers all IBDV strains (cv, ca, va, vv and d) (Martin *et al.*, 2007; Wu *et al.*, 2007; Xia *et al.*, 2008; Kim *et al.*, 2010; Hernández *et al.*, 2015). However, this methodology is time-consuming, expensive and requires trained staff, not being suitable to be widely applied in clinical settings. A good alternative is the application of molecular typing techniques (e.g. restriction fragment length polymorphisms and allele-specific PCR) that allow the straightforward virus classification by identifying nucleotide or amino acid residues specific for each IBDV strain. Several useful assays have been described for ca, va and vv strains (Peters *et al.*, 2005; Kong *et al.*, 2009; Ghorashi *et al.*, 2011; Hernández *et al.*, 2011; Tomás *et al.*, 2012), but no specific typing technique has been reported for the dIBDVs. Here, we present the development of a reverse transcription-quantitative PCR (RT-qPCR) assay for the specific detection of dIBDVs in the poultry industry worldwide.

## Materials and methods

### Sequence analysis and primer/probe design

Multiple sequence alignments were carried out with most of the hvVP2 sequences available in the GenBank database ($n = 955$) using the MUSCLE algorithm implemented in *MEGA* 5.0 (Tamura *et al.*, 2011). The dIBDV sequences were identified by phylogenetic clustering and amino acid markers (T272, P289, I290 and F296) following Hernández *et al.* (2015). Based on the nucleotide variants linked to the dIBDVs, specific primers and TaqMan-minor groove binding (TaqMan-MGB) probe were designed and synthesized by IDT DNA (Coralville, IA, USA) and Applied Biosystems (Foster City, CA, USA), respectively (Table 1). A BLAST search was also performed to predict in silico primer and probe sequence specificity in order to evaluate the occurrence of non-specific homology with other IBDV genome regions or with the chicken genome.

### dIBDV samples

The Uruguayan dIBDV field strain UY-221201 was used to generate the standard RNA transcripts for the standardization and testing of the analytical

**Table 2.** IBDV field samples and vaccine strains used in this study.

| Strain name | Genetic lineage | Sample type | Origin | Ct value[d] |
|---|---|---|---|---|
| UY-221201 | Distinct | Bursa | Uruguay | 22.3 |
| UY-42/07 | Distinct | Bursa | Uruguay | 30.3 |
| UY-04/09 | Distinct | Bursa | Uruguay | 22.9 |
| UY-04/10 | Distinct | Bursa | Uruguay | 23.8 |
| UY-06/10 | Distinct | Bursa | Uruguay | 23.6 |
| UY-07/10A | Distinct | Bursa | Uruguay | 24.0 |
| UY-07/10B | Distinct | Bursa | Uruguay | 16.6 |
| UY-07/10C | Distinct | Bursa | Uruguay | 18.8 |
| UY-07/10D | Distinct | Bursa | Uruguay | 19.5 |
| UY-171101 | Distinct | Bursa | Uruguay | 17.8 |
| UY-421101 | Distinct | Bursa | Uruguay | 16.1 |
| UY-421102 | Distinct | Bursa | Uruguay | 21.7 |
| UY-421103 | Distinct | Bursa | Uruguay | 20.7 |
| UY-221201 | Distinct | Bursa | Uruguay | 22.3 |
| UY-271201 | Distinct | Bursa | Uruguay | 17.1 |
| UY-301201 | Distinct | Bursa | Uruguay | 28.8 |
| UY-341201 | Distinct | Bursa | Uruguay | 26.6 |
| UY-351201 | Distinct | Bursa | Uruguay | 31.3 |
| UY-141403 | Distinct | Bursa | Uruguay | 28.1 |
| UY-171401 | Distinct | Bursa | Uruguay | 25.7 |
| dIBDV/UY/2014/2202 | Distinct | Bursa | Uruguay | 25.6 |
| UY-221401 | Classic virulent | Bursa | Uruguay | 40.0 |
| Winterfield 2512[a] | Classic virulent | Vaccine | – | 40.0 |
| Lukert[b] | Classic virulent | Vaccine | – | 40.0 |
| D78[c] | Classic attenuated | Vaccine | – | 40.0 |
| UY-281301 | Classic attenuated | Bursa | Uruguay | 40.0 |
| UY-291301 | Classic attenuated | Bursa | Uruguay | 40.0 |
| UY-251501 | Classic attenuated | Bursa | Uruguay | 40.0 |
| 1355 | Variant (Del-E) | Bursa | United States | 40.0 |
| 2564 | Variant (Del-E) | Bursa | United States | 40.0 |
| 2566 | Variant (Del-E) | Bursa | United States | 40.0 |
| 2567 | Variant (Del-E) | Bursa | United States | 40.0 |
| Uy-1 | Very virulent | Bursa | Uruguay | 40.0 |
| Uy-2 | Very virulent | Bursa | Uruguay | 40.0 |
| Uy-3 | Very virulent | Bursa | Uruguay | 40.0 |
| Uy-4 | Very virulent | Bursa | Uruguay | 40.0 |
| Uy-5 | Very virulent | Bursa | Uruguay | 40.0 |

[a]Obtained from the CEVAC-IBD-L vaccine, Ceva-Phylaxia, Budapest, Hungary.
[b]Obtained from the Bursine-2 vaccine, Fort Dodge Animal Health, Iowa, United States.
[c]Obtained from the NobilisGumboro D78 vaccine, Intervet International B.V., Boxmeer, Holland.
[d]Mean Ct value of two replicas. Threshold value = 0.05ΔRn.

performance of the developed RT-qPCR assay (Table 2). Twenty Uruguayan dIBDV outbreaks were employed to test the clinical sensitivity of the developed RT-qPCR assay (Table 2). Molecular diagnosis of the strains were performed by quantitative PCR (Tomás *et al.*, 2012), and assigned to the dIBDV lineage by hvVP2 sequence analysis (Hernández *et al.*, 2015).

### IBDV-negative samples

Thirty IBDV-negative field samples, diagnosed by quantitative PCR (Tomás *et al.*, 2012), were used for testing cross-reactivity (specificity).

### Avian viruses for specificity testing

Representatives of all IBDV strains (Table 2), and the following avian viruses were employed to assess the cross-reactivity of the assay: infectious bronchitis virus (Bronchitis vaccine Mass. Type, Fort Dodge Animal Health, IA, USA), avian reovirus (Tenosynovitis vaccine, Fort Dodge Animal Health, IA, USA), chicken infectious anaemia virus (Nobilis CAV P4 vaccine, Intervet International B.V., Boxmeer, Holland) and Newcastle diseases virus (Nobilis ND Hitchner vaccine, from Intervet International B.V).

### RNA extraction from vaccine virus and field samples

Total RNA was extracted using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA). RNA from vaccines was extracted using 200 µl of a phosphate-buffered saline resuspension of lyophilized vaccine. Tissue-infected samples were processed starting with 50 mg of bursae internal folds. The extracted RNA was eluted in 35 µl of RNase-free water.

### Reverse transcription and quantitative PCR assay

For the RT step, we used the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences Inc., Hanover, MD, USA) with 10 µl of extracted RNA. The whole IBDV genome was reverse transcribed to complementary DNA (cDNA) using random hexamer primers.

Quantitative PCR was carried out in a 20 µl reaction volume containing 1 × Hot Rox Master Mix (Bioron, Ludwigshafen, Germany), 300 nM each primer, 400 nM probe and 1 µl of cDNA. Thermocycling was performed on the ABI Prism 7500 (Applied Biosystems) and consisted of a 5 min hold stage at 50°C, followed by a 10 min denaturation at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, ending with a 5 min at 70°C final step. Fluorescent measurements were collected at the hold stage, at the 60°C step of each cycle, and at the end of the run.

### Generation of standard RNA for analytical testing

Standard RNA generation procedure was carried out as described by Tomás *et al.* (2012), with minor modifications. Briefly, a 491 bp genomic fragment encompassing the RT-qPCR amplicon was obtained from the UY-221201 dIBDV strain using P3- and P4-specific primers (Liu *et al.*, 1998). This amplicon was gel-purified and cloned into a pJET1.2 vector (Fermentas Life Sciences Inc.). Recovered plasmids were linearized and used for *in vitro* transcription with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas Life Sciences Inc.). Generated RNA transcripts were purified and the concentrations of the products were quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Waltham, MA), determining the average concentration after five measures. The copy number of RNA molecules was obtained by the following formula: $Y$ (RNA copies/µl) = [$X$ (g/µl) RNA/(nt transcript length × 340)] × $6.022 \times 10^{23}$ (Ummul Haninah *et al.*, 2010). RNA transcripts were diluted to obtain a $10^9$ copies/µl stock solution and stored at −80°C.

### Standard curve generation for analytical testing

A standard curve was generated using 10-fold serial dilutions containing $10^0$–$10^8$ RNA copies/µl. Each dilution was spiked in total RNA extracts (750 ng/µl) from IBDV-negative bursae to simulate a field sample environment, and analysed in triplicates in three independent runs by RT-qPCR. A standard curve was obtained by plotting threshold cycle (Ct) values per three replicates per standard dilution versus the logarithm of the RNA copy. Efficiency (E), coefficient of determination ($R^2$) and coefficients of variation (CV) were calculated from the resulting standard curves.

## Results

### Identification of dIBDVs in public databases

We detected 110 sequences in the hvVP2 dataset ($n$ = 955) that cluster within the dIBDV lineage and have the typical amino acidic signature T272, P289, I290 and F296. These dIBDV sequences came from 11 different countries (Argentina, Brazil, Canada, Colombia, Hungary, Poland, Puerto Rico, Russia, South Korea, the United States and Uruguay), and had been collected during 1977–2014 (Table S1, supplemental data).

One Argentine and six Brazilian sequences cluster within the dIBDV lineage but have a valine (V) instead of an isoleucine (I) in the residue 290 used as a marker;

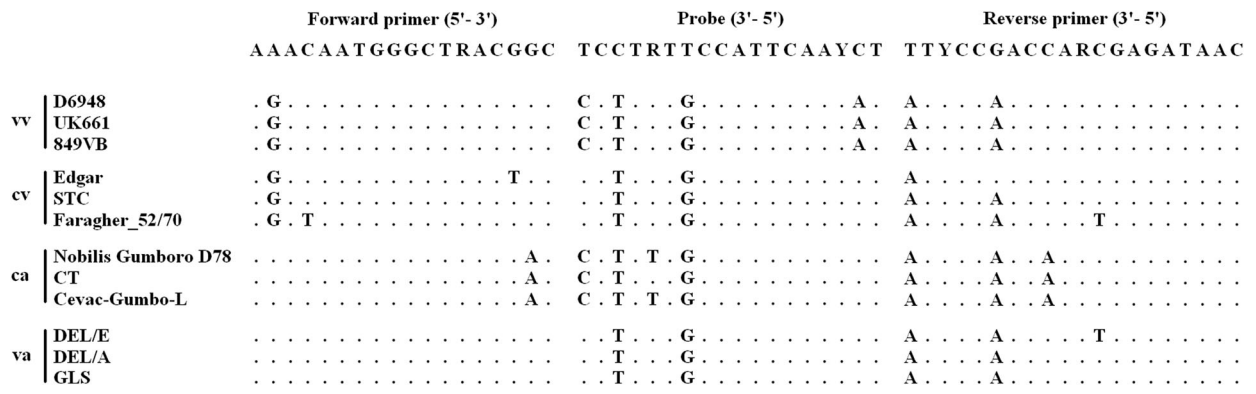


**Figure 1.** Alignment of primers and probe-target sites including sequences of different IBDV representative strains. Nucleotide residues equal to dIBDVs' specific primers and probe are indicated with dots. vv: very virulent; cv: classic virulent; ca: classic attenuated; va: variant.

this change is not present in any other IBDV sequence. We considered these sequences as belonging to dIBDVs with a slightly different amino acidic signature (T272, P289, *V290*, F296).

### *Assay design*

After identification of the strains, we focused our analysis on finding regions with optimal conditions for primer and probe design. The probe was designed to target a nucleotide signature that was exclusively found in the dIBDVs (Figure 1). Since the probe included the codon of the amino acid position 290, a degenerated nucleotide was included in the probe to detect all dIBDVs, regardless of the 290 residue (I or V). The designed TaqMan-MGB probe contains a minimum of two mismatches with non-dIBDV sequences, avoiding cross-reaction (Figure 1).

Primers were designed to match perfectly with the dIBDVs and to maximize the divergence with non-IBDV viruses, including attenuated vaccine strains. Both primers have some mismatches with other non-dIBDV strains, a 3′ mismatch in the reverse primer being the most relevant (Figure 1).

Nucleotide BLAST search of primers and probe showed only complete homology with dIBDV sequences. No cross-reaction signal was observed when a dIBDV-specific probe was tested with c, va and vvIBDV strains or with infectious bronchitis virus, avian reovirus, chicken infectious anaemia virus and Newcastle disease virus. No increase of fluorescent signal was detected in any case, resulting in Ct values equal to 40 (Table 2).

*Analytical performance of the assay*. A genomic fragment encompassing the RT-qPCR amplicon was successfully cloned and *in vitro* transcribed to obtain dIBDV RNA. Dilutions in total RNA extracts were analysed by the developed RT-qPCR. A standard curve was generated using 10-fold serial dilutions of RNA standards from $10^0$ to $10^8$ RNA copies/reaction. The linear dynamic range was established between $10^3$ to $10^8$ RNA copies/reaction, with an average $R^2$ value of 0.9991, and an efficiency of 91% (Figure 2).

The assay reproducibility assessed by the intra- and inter-assay CVs was lower than 3%.

*Clinical sensitivity of the assay*. All previously diagnosed and characterized dIBDV field strains were correctly diagnosed with the RT-qPCR assay. The fluorescent signal clearly increased above the threshold in all cases, showing Ct values ranging from 16.1 to 31.3 (Table 2). Probe and primer binding sites in these Uruguayan strains are completely conserved among global strains, indicating that the same clinical sensitivity would occur with the dIBDV viruses from different origins.

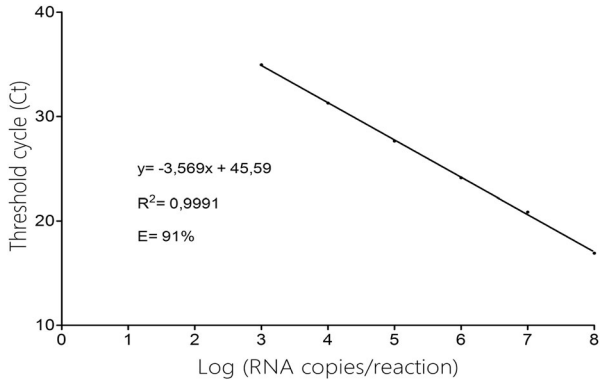


**Figure 2.** Standard curve of the developed RT-qPCR assay using the dIBDV-specific probe. Linear dynamic range was established between $10^3$ and $10^8$ RNA copies/reaction. Each point represents the mean Ct of nine different measures (three independent reactions, three replicates each). The coefficient of determination ($R^2$) and the efficiency (E) of the linear regression curve are indicated.

## Discussion

Assessing the current global prevalence and relevance of the recently described dIBDV lineage is of crucial importance for improving disease control and

understanding viral dynamics. The dIBDVs have been collected over a period of almost 40 years from different continents, an indicative of its wide and persistent spreading (Table S1). By analysing the hvVP2 sequences available in the GenBank, we inferred that more than 10% of the IBDV sequences correspond to dIBDVs, suggesting a high frequency of this lineage in the global virus population. Countries such as Argentina, Canada and Uruguay have reported a high prevalence of this lineage circulating in the poultry production, while in countries such as Brazil, Colombia, Hungary, Poland, Puerto Rico, Russia, South Korea and the United States, there are only sporadic reports of dIBDVs (Shcherbakova *et al.*, 1998; Kwon *et al.*, 2000; Ikuta *et al.*, 2001; Jackwood *et al.*, 2001; Smiley & Jackwood, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007; Hernández *et al.*, 2015; Tomás *et al.*, 2015; Vera *et al.*, 2015). This uneven prevalence among different countries needs to be confirmed by performing more extensive studies with a specific diagnostic method, taking into consideration that dIBDVs can be easily ignored during routine surveillance due to the apparent lack of differential clinical signs (Ikuta *et al.*, 2001; Domanska *et al.*, 2004).

The development of a specific and rapid method for dIBDVs differentiation requires the detailed analysis of the genetic variability of the virus. The hvVP2 region has a level of variability that allows the proper classification of all strains and the dIBDV lineage (Islam *et al.*, 2001; Le Nouën *et al.*, 2005; Yamaguchi *et al.*, 2007; Kim *et al.*, 2010; Liu *et al.*, 2013; Amin & Jackwood, 2014; Hernández *et al.*, 2015). The phylogenetic clustering and the molecular signature T272, P289, I290 and F296 were strongly conserved among the sequences despite being collected over a period of almost four decades. A few dIBDV isolates show an amino acid substitution in the 290 residue of the signature (I→V). Considering that V290 is also unique for the dIBDVs, the amino acidic signature T272, P289, I/V290 and F296 should be regarded as the right marker for the dIBDV lineage.

Here we developed an RT-qPCR assay for the rapid detection and precise identification of dIBDVs. The method bases its detection capacity on the presence of various molecular markers within the hvVP2 of dIBDV. The designing of primers and probes in the hvVP2 is challenging by the occurrence of single nucleotide polymorphisms in the region, implying that the target has to be carefully selected to include strong molecular markers that persist in time. In the probe hybridization region, two nucleotides are strongly conserved in all dIBDVs and located near the 3′ end of the probe, improving its discrimination capability and reinforcing the specificity of the assay (Figure 1) (Kutyavin *et al.*, 2000). These nucleotides are part of the codons for the P289 and I/V290 residues that comprise the amino acid signature of the dIBDV linage. The 289 and 290 amino acids are important because they occur within the hvVP2 hydrophilic peak 2, which is antigenically relevant in IBDV (Berg *et al.*, 1996). As these two nucleotides are not present in any other IBDVs, the designed TaqMan-MGB probe is expected to be highly specific; TaqMan-MGB probes form extremely stable duplexes with complementary DNA, and a single mismatch in the probe-target duplex would result in a high Tm difference that prevents cross-hybridization to non-specific targets (Kumar *et al.*, 1998). There are also mismatches between primers and target sites of non-dIBDVs (Figure 1). The most significant change occurs in the reverse primer that differs in its 3′ end with all known non-dIBDVs; this change reduces significantly the primer hybridization with non-dIBDVs and thus increases the specificity of the assay (Kwok *et al.*, 1990).

The RT-qPCR assay was assessed by testing its analytical performance, specificity and clinical sensitivity. Analytical tests indicate that the assay has a broad linear dynamic range ($10^3$–$10^8$ RNA copies/reaction), and acceptable coefficient of determination and PCR efficiency (0.9991 and 91%, respectively) (Figure 2). The assay also shows high specificity and lack of cross-reactivity with non-dIBDV strains, IBDV-negative samples and other common avian viruses. All dIBDV samples tested positive with this assay, which in conjunction with the high detection capacity ($10^3$ genome copies/reaction) support its use as a good diagnostic method, even in samples with low viral titre.

Another main application of the assay is the precise quantification of viral genomes in field samples. This could be used for establishing the virus load in diverse tissues and to provide information about the intra-host circulation. In protection assays, the accurate identification and quantification of the field strain is essential and has to be distinguished from the vaccine virus, which might replicate and persist in the bursa of Fabricius for several days (Ashraf *et al.*, 2005; Iván *et al.*, 2005). It is also possible that different strains co-infect the same avian host, making mapping the quantification and distribution of the viruses relevant (Stoute *et al.*, 2013).

The possibility of screening a large number of samples in a rapid, sensitive and reproducible way makes this assay a suitable tool for dIBDV impact assessment in field samples. It could be used to simultaneously diagnose and characterize dIBDV samples with low titres, since the high sensitivity of the assay facilitates the detection of few genome copies, allowing the direct analysis of the virus without prior propagating in culture or in embryonated eggs. It is expected that these attributes improve the IBDV surveillance and control worldwide and increase our understanding

of the molecular epidemiology of this economically detrimental poultry pathogen.

## Acknowledgments

We thank Granjas Hepa Ltda. for collaboration.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by the Instituto Nacional de Investigación Agropecuaria (INIA – Uruguay) under Project number 319 of the Fondo de Promoción de Tecnología Agropecuaria; Comisión Sectorial de Investigación Científica; Programa de Desarrollo de las Ciencias Básicas; and Agencia Nacional de Investigación e Innovación.

## References

Amin, O.G.M. & Jackwood, D.J. (2014). Identification and molecular analysis of infectious bursal disease in broiler farms in the Kurdistan Regional Government of Iraq. *Tropical Animal Health and Production*, 46, 1297–1301.

Ashraf, S., Abdel-Alim, G., Al-Natour, M.Q. & Saif, Y.M. (2005). Interference between mild and pathogenic strains of infectious bursal disease virus in chickens. *Avian Diseases*, 49, 99–103.

Berg, T.P., Gonze, M., Morales, D. & Meulemans, G. (1996). Acute infectious bursal disease in poultry: immunological and molecular basis of antigenicity of a highly virulent strain. *Avian Pathology*, 25, 751–768.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology*, 76, 2393–2402.

Domanska, K., Mato, T., Rivallan, G., Smietanka, K., Minta, Z., de Boisseson, C., Toquin, D., Lomniczi, B., Palya, V. & Eterradossi, N. (2004). Antigenic and genetic diversity of early European isolates of infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of infectious bursal disease virus revisited? *Archives of Virology*, 149, 465–480.

Fahey, K.J., Chapman, A.J., Macreadie, I.G., Vaughan, P.R., McKern, N.M., Skicko, J.I., Ward, C.W. & Azad, A.A. (1991). A recombinant subunit vaccine that protects progeny chickens from infectious bursal disease. *Avian Pathology*, 20, 447–460.

Ghorashi, S.A., O'Rourke, D., Ignjatovic, J. & Noormohammadi, A.H. (2011). Differentiation of infectious bursal disease virus strains using real-time RT-PCR and high resolution melt curve analysis. *Journal of Virological Methods*, 171, 264–271.

Hernández, M., Tomás, G., Hernández, D., Villegas, P., Banda, A., Maya, L., Panzera, Y. & Pérez, R. (2011). Novel multiplex RT-PCR/RFLP diagnostic test to differentiate low- from high-pathogenic strains and to detect reassortant infectious bursal disease virus. *Avian Diseases*, 55, 368–374.

Hernández, M., Tomás, G., Marandino, A., Iraola, G., Maya, L., Mattion, N., Hernández, D., Villegas, P., Banda, A., Panzera, Y. & Pérez, R. (2015). Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. *Avian Pathology*, 44, 212–221.

Ikuta, N., El-Attrache, J., Villegas, P., García, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. & Marques, E.K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Diseases*, 45, 297–306.

Islam, M.R., Zierenberg, K. & Muller, H. (2001). The genome segment B encoding the RNA-dependent RNA polymerase protein VP1 of very virulent infectious bursal disease virus (IBDV) is phylogenetically distinct from that of all other IBDV strains. *Archives of Virology*, 146, 2481–2492.

Ismail, N.M., Saif, Y.M. & Moorhead, P.D. (1988). Lack of pathogenicity of five serotype 2 infectious bursal disease viruses in chickens. *Avian Diseases*, 32, 757–759.

Iván, J., Velhner, M., Ursu, K., German, P., Mató, T., Drén, C.N. & Mészáros, J. (2005). Delayed vaccine virus replication in chickens vaccinated subcutaneously with an immune complex infectious bursal disease vaccine: quantification of vaccine virus by real-time polymerase chain reaction. *Canadian Journal of Veterinary Research*, 69, 135–142.

Jackwood, D.J., Sommer, S.E. & Knoblich, H. V. (2001). Amino acid comparison of infectious bursal disease viruses placed in the same or different molecular groups by RT/PCR-RFLP. *Avian Diseases*, 45, 330–339.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal disease viruses from four continents. *Virology*, 365, 369–375.

Kim, H.-R., Kwon, Y.-K., Bae, Y.-C., Oem, J.-K. & Lee, O.-S. (2010). Genetic characteristics of virion protein 2 genes of infectious bursal disease viruses isolated from commercial chickens with clinical disease in South Korea. *Poultry Science*, 89, 1642–1646.

Kong, L.L., Omar, A.R., Hair Bejo, M., Ideris, A. & Tan, S.W. (2009). Development of SYBR green I based one-step real-time RT-PCR assay for the detection and differentiation of very virulent and classical strains of infectious bursal disease virus. *Journal of Virological Methods*, 161, 271–279.

Kumar, S., Reed, M.W., Gamper, H.B., Gorn, V.V., Lukhtanov, E.A., Foti, M., West, J., Meyer, R.B. & Schweitzer, B.I. (1998). Solution structure of a highly stable DNA duplex conjugated to a minor groove binder. *Nucleic Acids Research*, 26, 831–838.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B. & Hedgpeth, J. (2000). 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research*, 28, 655–661.

Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C. & Sninsky, J.J. (1990). Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Research*, 18, 999–1005.

Kwon, H.M., Kim, D.K., Hahn, T.W., Han, J.H. & Jackwood, D.J. (2000). Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. *Avian Diseases*, 44, 691–696.

Le Nouën, C., Rivallan, G., Toquin, D. & Eterradossi, N. (2005). Significance of the genetic relationships deduced from partial nucleotide sequencing of infectious bursal disease virus genome segments A or B. *Archives of Virology*, 150, 313–325.

Lejal, N., Costa, B.D., Huet, J.C., Delmas, B. & Da Costa, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology*, 81, 983–992.

Liu, X., Giambrone, J.J. & Dormitorio, T. (1998). Simplified sample processing combined with a sensitive nested polymerase chain reaction assay for detection of infectious bursal disease virus in the bursa of Fabricus. *Avian Diseases*, 42, 480–485.

Liu, D., Zhang, X.-B., Yan, Z.-Q., Chen, F., Ji, J., Qin, J.-P., Li, H.-Y., Lu, J.-P., Xue, Y., Liu, J.-J., Xie, Q.-M., Ma, J.-Y., Xue, C.-Y. & Bee, Y.-Z. (2013). Molecular characterization and phylogenetic analysis of infectious bursal disease viruses isolated from chicken in South China in 2011. *Tropical Animal Health and Production*, 45, 1107–1112.

Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodríguez, J.F. & Castón, J.R. (2009). Infectious bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *Journal of Molecular Biology*, 386, 891–901.

Martin, A.M., Fallacara, F., Barbieri, I., Tosi, G., Rivallan, G., Eterradossi, N., Ceruti, R. & Cordioli, P. (2007). Genetic and antigenic characterization of infectious bursal disease viruses isolated in Italy during the period 2002-2005. *Avian Diseases*, 51, 863–872.

Müller, H., Scholtissek, C. & Becht, H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. *Journal of Virology*, 31, 584–589.

Ojkic, D., Martin, E., Swinton, J., Binnington, B. & Brash, M. (2007). Genotyping of Canadian field strains of infectious bursal disease virus. *Avian Pathology*, 36, 427–433.

Peters, M.A., Lin, T.L. & Wu, C.C. (2005). Real-time RT-PCR differentiation and quantitation of infectious bursal disease virus strains using dual-labeled fluorescent probes. *Journal of Virological Methods*, 127, 87–95.

Remorini, P., Calderón, M.G., Aguirre, S., Periolo, O., La Torre, J. & Mattion, N. (2006). Characterization of infectious bursal disease viruses from Argentina. *Avian Diseases*, 50, 245–251.

Rosenberger, J.K. & Gelb, J. (1978). Response to several avian respiratory viruses as affected by infectious bursal disease virus. *Avian Diseases*, 22, 95–105.

Sánchez, A.B., Rodriguez, J.F. & Sanchez, A.B. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology*, 262, 190–199.

Shcherbakova, L.O., Lomakin, A.I., Borisov, A.V., Drygin, V.V. & Gusev, A.A. (1998). Comparative analysis of the VP2 variable region of the gene from infectious bursal disease virus isolates. *Molekuliarnaia Genetika, Mikrobiologiia i Virusologiia*, 1,35–40.

Smiley, J.R. & Jackwood, D.J. (2001). Genetic stability of the VP2 hypervariable region of four infectious bursal disease virus isolates after serial passage in specific-pathogen-free chicken embryos. *Avian Diseases*, 45, 1–8.

Spies, U., Müller, H. & Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Research*, 8, 127–140.

Stoute, S.T., Jackwood, D.J., Sommer-Wagner, S.E., Crossley, B.M., Woolcock, P.R. & Charlton, B.R. (2013). Pathogenicity associated with coinfection with very virulent infectious bursal disease and Infectious bursal disease virus strains endemic in the United States. *Journal of Veterinary Diagnostic Investigation*, 25, 352–358.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Tomás, G., Hernández, M., Marandino, A., Hernández, D., Techera, C., Grecco, S., Panzera, Y. & Pérez, R. (2015). Genome sequence of a distinct infectious bursal disease virus. *Genome Announcements*, 3, e01061–15.

Tomás, G., Hernández, M., Marandino, A., Panzera, Y., Maya, L., Hernández, D., Pereda, A., Banda, A., Villegas, P., Aguirre, S. & Pérez, R. (2012). Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. *Journal of Virological Methods*, 185, 101–107.

Ummul Haninah, A., Vasan, S.S., Ravindran, T., Chandru, A., Lee, H.L. & Shamala Devi, S. (2010). Development and evaluation of a one-step SYBR-Green I-based real-time RT-PCR assay for the detection and quantification of Chikungunya virus in human, monkey and mosquito samples. *Tropical Biomedicine*, 27, 611–623.

Van Den Berg, T.P., Eterradossi, N., Toquin, D. & Meulemans, G. (2000). Infectious bursal disease (Gumboro disease). *Revue Scientifique et Technique International Office of Epizootics*, 19, 509–543.

Vera, F., Craig, M.I., Olivera, V., Rojas, F., König, G., Pereda, A. & Vagnozzi, A. (2015). Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. *Archives of Virology*, 160, 1909–1921.

Wu, C.C., Rubinelli, P. & Lin, T.L. (2007). Molecular detection and differentiation of infectious bursal disease virus. *Avian Diseases*, 51, 515–526.

Wu, Y., Hong, L., Ye, J., Huang, Z. & Zhou, J. (2009). The VP5 protein of infectious bursal disease virus promotes virion release from infected cells and is not involved in cell death. *Archives of Virology*, 154, 1873–1882.

Xia, R.X., Wang, H.Y., Huang, G.M. & Zhang, M.F. (2008). Sequence and phylogenetic analysis of a Chinese very virulent infectious bursal disease virus. *Archives of Virology*, 153, 1725–1729.

Yamaguchi, T., Kasanga, C.J., Terasaki, K., Maw, M.T., Ohya, K. & Fukushi, H. (2007). Nucleotide sequence analysis of VP2 hypervariable domain of infectious bursal disease virus detected in Japan from 1993 to 2004. *The Journal of Veterinary Medical Science*, 69, 733–738.


# Origin and global spreading of an ancestral lineage of the infectious bursal disease virus

| | |
|---|---|
| Journal: | *Transboundary and Emerging Diseases* |
| Manuscript ID | TBED-OA-703-19.R2 |
| Manuscript Type: | Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Tomás, Gonzalo; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República,<br>Marandino, Ana; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República,<br>Techera, Claudia; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República<br>Olivera, Valeria; Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, 1686<br>Perbolianachis, Paula; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República<br>Fuques, Eddie; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República<br>Grecco, Sofía; Universidad de la República Uruguay, Facultad de Ciencias<br>Hernández, Martín; Facultad de Ciencias, Sección Genética Evolutiva<br>Hernández, Diego; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República<br>Calleros, Lucía; Facultad de Ciencias, Sección Genética Evolutiva<br>Craig, María; Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, 1686<br>Panzera, Yanina; Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República<br>Vagnozzi, Ariel; Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, 1686 Buenos Aires, AR<br>Pérez, Ruben; Facultad de Ciencias, Sección Genética Evolutiva |
| Subject Area: | IBDV, evolution, phylodynamics, distinct genogroup |
| | |

TITLE: **Origin and global spreading of an ancestral lineage of the infectious bursal disease virus**

SHORT RUNNING TITLE: **Origin and spreading of IBDV**

Gonzalo Tomás[1], Ana Marandino[1], Claudia Techera[1], Valeria Olivera[2], Paula Perbolianachis[1], Eddie Fuques[1], Sofía Grecco[1], Martín Hernández[1], Diego Hernández[1], Lucía Calleros[1], María Isabel Craig[2], Yanina Panzera[1], Ariel Vagnozzi[2] & Ruben Pérez[1*].

[1]Sección Genética Evolutiva, Departamento de Biología Animal, Instituto de Biología, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay.

[2]Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, 1686, Buenos Aires, Argentina.

[*]To whom correspondence should be addressed.

Tel: +598 2 525 8618; fax: +598 2 525 8617. E-mail address: rperez@fcien.edu.uy

## SUMMARY

Infectious bursal disease virus (IBDV) is an economically relevant and widespread pathogen that produces immunosuppression in young chickens. IBDV is genetically classified into seven genogroups (G1 to G7), where the traditional classic, variant and very virulent strains correspond to G1, G2 and G3, respectively. The G4 strains, also known as "distinct" (dIBDV), have recently acquired increased relevance because of their prevalence and notorious impair to the poultry industry in South America. Here, worldwide dIBDV strains were studied using phylogenetic and phylodynamic approaches. The phylogenetic analyses performed using partial and complete sequences of both viral segments (A and B) consistently clustered the dIBDV strains in a monophyletic group. The analyses of the VP5, polyprotein and VP1 coding regions identified amino acid residues that act as markers for the identification of the entire dIBDV group or different sub-populations. The phylodynamic analyses performed using the hypervariable region of VP2 indicated that the dIBDV strains emerged in the early 1930s in Eastern Europe, shortly after the emergence of classic strains (1927) and before variant (1949) and very virulent strains (1967). The analysis of the migration routes indicated that after its emergence, the dIBDV strains spread to Eastern Asia around 1959, to Brazil around 1963, and to Argentina around 1990. These intercontinental migrations resulted in three sub-populations that are currently represented by strains from (i) Brazil, (ii) Eastern Asia and Canada, and (iii) Eastern Europe, Argentina and Uruguay. Taken together, our results highlight the complex evolutionary history of IBDV and the importance of new phylodynamic data to unravel and nearly follow the different evolutionary pathways taken by this important poultry pathogen.

Keywords: IBDV, evolution, phylodynamics, distinct genogroup.

## Introduction

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease that affects young chickens. The virus replicates in the bursa of Fabricius and other lymphoid tissues resulting in immunosuppression, which leads to an increased susceptibility to other infectious agents and a lack of humoral response to vaccinations (Allan et al., 1972; Faragher et al., 1974; Rosenberger & Gelb, 1978). IBDV infections are widespread and have a severe economic impact on the commercial poultry production (McIlroy et al., 1989; Zachar et al., 2016).

IBDV belongs to the genus *Avibirnavirus* within the family *Birnaviridae*, which is characterized by a non-enveloped icosahedral capsid and two double stranded RNA genome segments, named A and B (Dobos et al., 1979; Muller et al., 1979). Genome segment A (3200 bp) encodes a polyprotein that is further processed into three viral proteins (VP2, VP3 and VP4) and four small peptides, and in a partially overlapped open reading frame the non-structural protein VP5 (Mundt et al., 1995; Sánchez & Rodriguez, 1999; Lejal et al., 2000; Da Costa et al., 2002; Luque et al., 2009). Genome segment B (2800 bp) encodes the viral RNA polymerase VP1 (Spies et al., 1987).

The IBDV genome evolves rapidly via mutation, and less commonly by reassortment of both genome segments and recombination (Le Nouen et al., 2006; He et al., 2009; Soubies et al., 2017; Pikula et al., 2018). The action of these mechanisms has led to the emergence of a great deal of genetic variability, as evidenced by the existence of diverse evolutionary lineages in the viral populations (Michel & Jackwood, 2017).

There are two IBDV serotypes distinguished by cross neutralization test, of which only serotype 1 viruses cause clinical disease in chickens (McFerran et al., 1980; Jackwood et al., 1985; Ismail et al., 1988). Serotype 1 was traditionally divided into classic (c), variant (va) and very virulent (vv) strains using antigenic and pathogenic criteria (Rosenberger et al., 1985; Chettle et al., 1989). Transferring this antigenic/pathogenic classification to a genetic-based nomenclature has been relatively successful for IBDV; genetic lineages are mostly concordant with traditional strains classification. Accordingly, most new isolates are currently only genetically classified but named using the antigenic/pathogenic nomenclature as cIBDV, vaIBDV and vvIBDV. Sequence analyses also detect reassortments, recombinants and new lineages that need to be considered for IBDV classification. A recent study assessed a comprehensive classification of all IBDV strains resulting in seven well-supported genetic lineages denoted as genogroups (G) 1 to 7 (Michel & Jackwood, 2017). According to this classification, the cIBDV, vaIBDV and vvIBDV strains correspond to G1, G2 and G3, respectively. Genogroups 4 to 7 include the ‘distinct’ (d) IBDV strains (G4), a group of proposed recombinant strains from Mexico (G5), divergent strains mainly from Italy (G6), and Australian strains (G7) (Ignjatovic & Sapats, 2002; Jackwood, 2012; Hernández et al., 2015; Lupini et al., 2016).

The dIBDV comprises highly divergent strains that were collected in the last four decades from South and North America, Asia and Europe (Shcherbakova et al., 1998; Kwon et al., 2000; Jackwood et al., 2001; Ikuta et al., 2001; Domanska et al., 2004; Jackwood & Sommer-Wagner, 2007; Ojkic et al., 2007; Jeon et al., 2009; Vera et al., 2015; Hernández et al., 2015; Yamazaki et al., 2017; Michel & Jackwood, 2017). These strains have a particular antigenic profile and produce a subclinical infection which results in a marked bursal atrophy and immunosuppression (Minta et al., 1985a, 1985b; Domanska et al., 2004; Jeon et al., 2009; Yamazaki et al., 2017; Tomás et al., 2019).

Although the dIBDV strains have been overlooked in the past, particularly by the lack of specific clinical signs, they have acquired increased relevance because of their prevalence and potential economic impact in South America (Argentine, Brazil and Uruguay) and North America (Canada) (Ikuta et al., 2001; Ojkic et al., 2007, Hernández et al., 2015; Vera et al., 2015). However, there is still little information about their genetic characteristics and spatio-temporal dynamics.

Here, the origin and global spreading of the dIBDV strains were studied using a phylodynamic approach. The time to the most recent common ancestor (tMRCA), routes of migration and genetic changes associated with spreading and local differentiation were obtained to provide new insights into the evolutionary epidemiology of this relevant IBDV genogroup.

## Materials and methods

### Sequencing of dIBDV strains

Argentine (n=13) and Uruguayan (n=11) dIBDV strains collected between 2009 and 2016 were used for partial or complete coding genome amplification (Table 1). Samples were diagnosed and genotyped using previously described RT-qPCR assays (Tomás et al., 2012; Tomás et al., 2017). For partial genome sequencing, 492- and 594-bp fragments corresponding to the hypervariable region of VP2 (hvVP2) and VP1 genes, respectively, were amplified using previously described primers and conditions (Tomás et al., 2019). The complete coding genome was obtained from six partially overlapped amplicons using primers and conditions detailed in Supporting information 1. All PCR amplicons were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research) and sent to Macrogen for bidirectional sequencing (Macrogen Inc, Seoul, South Korea). Obtained sequences were edited and assembled using the SeqMan Pro software v15.0.1 (DNASTAR).

### Datasets

To assess the different analyses, both partial and complete datasets of genome segments A and B were generated using sequences available in the Genbank and those obtained in the present study.
The VP2 and VP1 datasets of partial sequences corresponded to a 356-nt fragment from segment A positions 795 to 1150, and a 533-nt fragment encompassing segment B positions 313 and 845. Regarding complete genome datasets, the regions corresponded to the full-coding segment A (position 97 to 3169) and B (position 112 to 2751). Numbering corresponds to segments A (AY029166) and B (AY029165) sequences of the strain Irwin Moulthrop.

### Sequence alignments and detection of recombinant sequences

Nucleotide alignments were carried out using the MUSCLE algorithm implemented in MEGA 7 (Kumar et al., 2016). A search of recombinant sequences was assessed with the complete genome datasets using the RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq methods, implemented in the RDP4 v4.95 software, using the default parameters and assuming recombination events if detected by at least four methods.

### Phylogenetic and amino acid analyses

Phylogenetic analyses were performed with both partial and complete genome datasets. In each case, the best-fit nucleotide substitution model was selected with the jModelTest2 software under the Akaike criterion (Darriba et al., 2012; Guindon & Gascuel, 2003). Maximum-likelihood phylogenetic trees were inferred using the PhyML v3.0 with approximate likelihood ratio test as internal nodes support (Guindon et al., 2010). The visualization and edition of the trees were carried out using the iTOL v4 online tool (Letunic & Bork, 2016).

The amino acid sequences corresponding to VP5, polyprotein and VP1 of all dIBDV strains were compared to the other strains using partial and complete coding sequences to identify genogroup specific markers for dIBDV. The term "marker" refers to a residue associated to a group of strains but whose biological implication may or may not be currently known.

**Phylodynamic analysis of the cIBDV, vaIBDV, vvIBDV and dIBDV strains**

A Bayesian Markov Chain Monte Carlo approach was implemented in the BEAST v1.8.4 package using hvVP2 sequences of the cIBDV, vaIBDV, vvIBDV and dIBDV strains; strains were selected to ensure a wide span of collection dates and geographic origins. The analysis did not include strains from the rest of genogroups (G5 to G7), vaccine or cell culture-adapted strains, nor recombinant or reassortant viruses. Sequence alignment and best-fit nucleotide substitution model were determined as detailed in the previous section. The TempEst v1.5.1 software was used to measure the temporal signal of the dataset. To determine which combination of molecular clock (strict, uncorrelated relaxed lognormal, uncorrelated relaxed exponential) and coalescent tree prior (constant, exponential, Bayesian skyline) best-fit to the dataset, the log marginal likelihoods were estimated through path and stepping stone sampling calculations. For each combination, two independent runs were performed consisting of $2 \times 10^8$ steps with sampling every $2 \times 10^4$ steps. Analyses were run using the BEAGLE library at the CIPRES Science Gateway server (Miller et al., 2010). Results were analyzed with TRACER v1.7 by evaluating the convergence of all estimators through the effective sampling size (ESS) after a 10% burn-in, considering convergence when ESS values were above 200.

**Phylogeographic analysis of the dIBDV strains**

A discrete trait phylogeographic analysis implemented in the BEAST v1.8.4 package was carried out using a subset of the hvVP2 sequences including all the dIBDV strains with known collection date and geographic origin. Sequence alignment, best-fit nucleotide substitution model, best-fit molecular clock and coalescent tree prior, and the measure of the temporal signal of the dataset were assessed as mentioned in previous sections. To minimize sampling bias, countries with low number of sequences were pooled together according to geographic closeness resulting in groups representing Eastern Europe (Hungary and Poland, n=5), Eastern Asia (Japan and South Korea, n=4) and Western Asia (Saudi Arabia and United Arab Emirates, n=3). Those countries represented by only one sequence and that could not be pooled according to the mentioned criteria (i.e. Russia, Puerto Rico and Colombia) were not included in the analysis. A non-reversible continuous-time Markov chain model was used, with a Bayesian stochastic search variable selection (BSSVS) approach to identify significant migratory events, assuming Bayes factor (BF) values above 3 as significant. Each run ($2 \times 10^8$ steps, sampling every $2 \times 10^4$ steps) was analyzed with TRACER v1.7. A maximum clade credibility tree (MCCT) was generated using the TreeAnotator v1.8.4 discarding 10% of states as burn-in, while the FigTree v1.4.3 was used for the visualization and edition of the tree. The resulting discrete phylogeographic analysis, particularly the support of each migration event, was

determined using the SPREAD software v1.0.7.

**Accession numbers.** The new sequences obtained in this study were submitted to Genbank and assigned accession numbers MN313610 to MN313648.

## Results

### Phylogenetic analyses

All the sequences detected as recombinants were excluded from the phylogenetic analyses (Supporting information 2).

The phylogenetic analysis of the hvVP2 region resolved the seven previously described genogroups (G1 to G7 in Figure 1). The newly obtained Argentine and Uruguayan field strains clustered with the other dIBDV strains as expected according to the result of the RT-qPCR assay. Serotype 2 strains occupied a basal position in the phylogenetic tree.

The phylogeny with partial sequences of the VP1 gene showed a well-defined clade comprising dIBDV strains and a sister single clade encompassing cIBDV, vaIBDV and one Italian G6 strain (Figure 2). Serotype 2, G7 and vvIBDV strains appeared as early-branching clades in the phylogenetic tree.

The analysis of the full-length genome sequences included only cIBDV, vaIBDV, vvIBDV and dIBDV from serotype 1 and strains from serotype 2 because there are not complete genome sequences available from any G5, G6 or G7 strains. The phylogeny with the segment A showed a well-supported clade corresponding to the dIBDV strains (Figure 3). The cIBDV, vaIBDV and vvIBDV strains were also separated in independent clades, while serotype 2 strains located in a basal position of the phylogeny. The segment B phylogeny yielded a well-resolved dIBDV clade that associated with a sister group of interspersed clustering of cIBDV and vaIBDV strains (Figure 3). The serotype 2 and vvIBDV strains occupied a completely different and early-branching position in the tree.

### Characterization of residues associated with the dIBDV strains

The VP5, polyprotein and VP1 sequences were inspected for the presence of marker residues associated with dIBDV strains (Table 2).

VP5 had the marker R135 that also occurred in two cIBDV strains, Edgar and CJ801, which were collected many years ago from USA and China, respectively. There were also residues that identify sub-populations within the dIBDV strains (sub-population specific residues), including P40 for Argentina and Uruguay and C137 for Brazil.

The polyprotein analysis showed six marker residues in VP2, two in VP3 and two in VP4. The VP2 markers corresponded to V124, S222, T272, P289, I290 and F296. The V124 residue also occurred in the Edgar and CJ801 strains, and the S222 residue was also present in some cIBDV vaccine strains, including the Lukert and 228E. The T272 residue was present in most dIBDV strains but was also characteristic of the G7 strains. The other markers (P289, I290 and F296) were unique for the dIBDV strains. The P5 and G245 were sub-

population specific residues for Argentine and Uruguayan dIBDV strains; the Hungarian and Polish strains, whose hvVP2 sequences were the only available, also had the G245 residue.
The VP4 coding region showed the dIBDV markers W680 and I711, while VP3 had the S777 and I922 markers.

In VP1, the D56 and S57 residues were markers strongly associated with the dIBDV strains. The S145, P243 and Q651 residues were sub-population specific markers for Argentina and Uruguay; P243 also occurred in the Polish dIBDV strains. The Brazilian dIBDV strains showed the sub-population specific residues D145, G305 and S849.

**Phylodynamic and phylogeographic analyses of the dIBDV strains**

The clock-likeness of the dataset was confirmed by the TempEst software, obtaining a correlation coefficient value of 0.3846. Path and stepping stone sampling analyses determined that the uncorrelated relaxed exponential clock with the exponential demographic model was the most accurate for the dataset (Supporting information 3). The most probable geographic origin of the dIBDV lineage was Eastern Europe (Poland and Hungary) in around 1933 (Figure 4). The first intercontinental migration event from Eastern Europe would have occurred around 1959 to Eastern Asia (Japan and South Korea) (BF = 4.3), from where it would have reached Canada by 1978 (BF = 19) (Figure 5). Its introduction in South America would have taken place in two different events; the first one to Brazil in approximately 1963 (BF = 2.3) and the second one to Argentina around 1990 (BF = 90). The arrival to Uruguay would have occurred through two independent migration events around 2005 – 2006 from Argentina (BF = 914). Western Asia (Saudi Arabia and United Arab Emirates) strains of dIBDV might appeared as a consequence of more recent intercontinental migration events from Brazil (BF = 137) and Canada (BF = 170).

**Evolutionary timeline of the cIBDV, vaIBDV, vvIBDV and dIBDV strains**

The origin dates of the major IBDV lineages were estimated. The TempEst analysis indicated that the dataset contained sufficient temporal signal, as evidenced by the positive correlation between genetic divergence and sampling time. However, the correlation coefficient value was 0.2378, with an outlier sequence corresponding to the only serotype 2 strain included (OH strain). After removing this sequence the correlation coefficient increased to 0.4173, thus indicating that an adequate temporal signal was present within the serotype 1 strains. The uncorrelated relaxed lognormal molecular clock with the bayesian skyline demographic model best fitted the dataset according to path and stepping stone sampling (Supporting information 3).
The tMRCA mean values for the cIBDV, vaIBDV, vvIBDV and dIBDV lineages were estimated to be 1927, 1949, 1967 and 1931, respectively (Table 3).

## Discussion

The evolutionary history of IBDV was characterized by the emergence of strains that diverged in their genetic composition and phenotypic features. The vaIBDV and vvIBDV strains focused the attention of the international community mainly due to the notorious impact to the poultry industry (Rosenberger et al., 1985; Rosales et al., 1989; Sharma et al., 1989; Chettle et al., 1989; van den Berg et al., 1991). Other strains, despite being problematic, were geographically restricted and less studied (e.g. the Australian strains) (Firth, 1974; Ignjatovic & Sapats, 2002). The dIBDV strains have been circulating in Europe, Asia, South and North America during many years but remain almost unnoticed, probably because they were not initially associated with a notorious impair to the poultry industry. Most dIBDV strains were reported as local variants and not as a unique evolutionary lineage (Jackwood & Sommer, 1997; Kwon et al., 2000; Ikuta et al., 2001; Banda et al., 2003; Remorini et al., 2006; Ojkic et al., 2007; Jeon et al., 2009; Yamazaki et al., 2017). This explains the scarce epidemiological data about the genomic characteristics, geographic origin and spatio-temporal spreading of dIBDV. Moreover, the truly relevance in the context of the general evolution of IBDV have not been discussed.

### Phylogenetic analyses

The phylogenetic analyses here assessed provide a comprehensive scenario of the evolutionary relationships between the dIBDV and the remaining genogroups. Interestingly, in all the phylogenies assessed with partial and complete genome sequences of segments A and B, the dIBDV strains consistently clustered in a monophyletic group clearly separated from the rest of strains (Figure 1, 2 and 3). This level of differentiation is commonly expected using the highly divergent hvVP2 region of segment A, but is unusual for other regions, as evidenced by the common clustering of the cIBDV and vaIBDV strains in phylogenies with either partial or complete sequences of segment B (Figure 2 and 3). The single-clustering observed with sequences of both dIBDV segments may be consequence of an accelerated evolutionary rate of the dIBDV segment B and/or a more ancient diversification that allowed the accumulation of nucleotide and amino acid substitutions. The evolutionary rate of the dIBDV segment B remains to be calculated but very few sequences are currently available to obtain a reliable estimation. On the other hand, the date of origin of the cIBDV, vaIBDV, vvIBDV and dIBDV strains was here estimated and the obtained results support the hypothesis of an ancient origin of the dIBDV strains (see below).

### Amino acid residues associated with the dIBDV strains

There are some residues along the segment A and B that act as markers for the characterization of the dIBDV genogroup. The most robust markers were those identified in the hvVP2 region because this sequence is usually obtained for IBDV characterization in diagnosis and research labs, and is thus over represented in the dataset.

The dIBDV markers detected in the hvVP2 region were S222, T272, P289, I290 and F296, which is in accordance with previous observations (Table 2) (de Fraga et al., 2019; Hernández et al., 2015; Vera et al., 2015). However, S222 and T272 occurred in some cIBDV vaccine strains and the G7 strains, respectively, and should then be considered less robust markers compared to P289, I290 and F296.

Other residues in VP1 (D56, S57), VP2 (V124), VP3 (S777, I922), VP4 (W680, I711) and VP5 (R135) were here detected as potential dIBDV markers as they were present in all the few dIBDV sequences that are currently available. To confirm these findings, more sequences of segment A and B have to be obtained, especially from Brazilian, Canadian or Asiatic dIBDV strains that are underrepresented in the database.

Interestingly, the residues V124 in VP2 and R135 in VP5 were also detected in the Edgar and CJ801 cIBDV strains. The Edgar strain collected in 1965 is one of the oldest IBDV strains described, while the CJ801 constitutes the first IBDV strain reported in China in 1979 (Zhou et al., 1981; Cao et al., 1998). Considering that no other cIBDV strain sequenced to date contain these residues, it is likely that they represent ancestral states that were lost during the evolution of the cIBDV lineage.

The dIBDV strains here analyzed have three different residues in the position 145 of VP1 (Table 2). This residue, together with residues 146 and 147, plays an important role in virus replication, pathogenicity and in the polymerase activity (Gao et al., 2014). Most vvIBDV strains have the signature TDN or TEG in position 145-147, while most cIBDV, vaIBDV and Polish dIBDV strains have the NEG residues. The presence of a different signature in Argentine and Uruguayan dIBDV strains (SEG), and in Brazilian strains (DEG), may have some biological implications that should be further analyzed.

**Epidemiological history of the dIBDV strains**

The phylodynamic and phylogeographic Bayesian inferences here assessed permitted the reconstruction of the early epidemiology of the major IBDV lineages and the migratory pattern of the dIBDV strains. Interestingly, the origin of the dIBDV lineage was set around the early 1930s in Eastern Europe, which is shortly after the estimated emergence of the cIBDV lineage in the late 1920s (Table 3). The European origin of the dIBDV strains is in accordance with the results obtained in a recently published study (de Fraga et al., 2019). Our findings indicate that both lineages (cIBDV and dIBDV) may have emerged almost at the same time in evolutionary terms, probably in different continents (North America and Europe). This supports a complex scenario that involved the existence of an ancestral IBDV circulating in different continents that underwent local differentiation to originate the widely spread cIBDV and dIBDV strains. Later, the cIBDV strains gave rise to the vaIBDV strains around 1949 and the vvIBDV strains around 1967, with the particularity that the segment B of the vvIBDV strains would have been acquired through a reassortment event (Hon et al., 2006). The estimated origin date of the vvIBDV strains is in accordance with previous reports; Hon et al. (2006) estimated the year of emergence of the vvIBDV strains around 1962 [95% HPD:

1950-1973], while Alfonso-Morales et al. (2013) estimated the same event to around 1970 [95% HPD: 1956-1985] (Hon et al., 2006; Alfonso-Morales et al., 2013). The date for the origin of the vaIBDV strains (around 1949) is here reported for the first time.

It does require an explanation of why disease signs were observed after some period of time from the putative origin of ancestral strains inferred from phylodynamic analysis. The first report of infectious bursal disease corresponded to cIBDV detected in Gumboro, Delaware, United States (Cosgrove, 1962), approximately three decades after the emergence of the putative ancestral cIBDV. Similarly, the reports of disease outbreaks caused by vaIBDV and vvIBDV occurred during the 1980s (Rosenberger et al., 1985; Chettle et al., 1989), two to three decades after the origin of the corresponding ancestral strains. The emergence of an IBDV strain as a disease causative agent requires two conditions to be fulfilled: i) the existence of a strain carrying the adequate genetic makeup, and ii) the appropriate, permissive ecological conditions (Geoghegan & Holmes, 2017). The proper genetic background could be achieved by the progressive accumulation of changes generated by point mutations, recombination and/or reassortment (Vakharia et al., 1994; Hon et al., 2006; Jackwood & Sommer-Wagner, 2007). These genetic changes are able to improve viral fitness (e.g. replication and transmission capacity) of an ancestral, less adapted strain that may have been circulating harmlessly in chickens or wild birds. To attain the ecological condition, the virus needs to adjust its relationship with the environment. IBDV ecology has been particularly altered in chicken after the 1950s as a consequence of the continuous expansion of the poultry industry, driven by changes in nutrition and chicken genetics, intensive rearing conditions, and selective pressures exerted by vaccination (Hewson, 1986; Gordon, 1996). This scenario may have provided the appropriate conditions for the virus to evolve and acquire new biological properties.

Regarding the dIBDV strains, three main successive intercontinental migration events were detected: (1) to Eastern Asia around 1959, (2) to Brazil around 1963, and (3) to Argentina around 1990 (Figure 4 and 5). This is concordant with the historical records indicating that Hungary and Poland were two of the main poultry producers and exporters in Europe from the early 1960s, which may have facilitated the introduction of dIBDV strains in other countries (FAOSTAT, 2018). Although the migration event from Eastern Europe to Brazil was supported by a slightly lower BF value (2.3), it is strongly supported by additional evidence. Silva et al. (2013) in a study of the IBDV epidemiology in Brazil identified migration events associated with strains currently known as dIBDV strains (e.g. MG8). Although they could not determine the geographic origin of these strains, the year of this migration event was estimated around 1969 (Silva et al., 2013), which is very close to the here obtained result (1963). The study by de Fraga et al. (2019) also support the existence of two intercontinental migration events from Eastern Europe to South America (Argentina and Brazil), and the introduction in Uruguay from Argentina in the middle 2000s.

The three migration events from Eastern Europe are concordant with the MCCT topology (Figure 4).

Accordingly, the dIBDV strains could be classified in three sub-populations composed mainly by strains from (1) Brazil, (2) Eastern Asia and Canada, and (3) Eastern Europe, Argentina and Uruguay. The recent occurrence of the migration event that introduced dIBDV strains in Argentina (around 1990) and later in Uruguay (around 2005-2006) may explain the similar characteristics shared with strains from Eastern Europe (Figure 5). On the contrary, those strains that migrated earlier as the Eastern Asian and Brazilian strains (1959 and 1963, respectively) had sufficient time to diverge and formed different sub-populations. This divergence is also evidenced by the presence of sub-population specific amino acid residues (Table 2). The Argentine and Uruguayan dIBDV strains have unique mutations in VP1 (S145, P243, Q651), VP2 (P5, G245) and VP5 (P40), while the Brazilian dIBDV strains have unique mutations in VP1 (D145, G305, S859) and VP5 (C137). A sub-clade of Brazilian strains also has the A275T and N280T substitutions that evidence an additional level of intra-country differentiation.

The introduction of the dIBDV strains in Western Asia countries may have recently occurred from Brazil and Canada (Figure 5). This hypothesis is supported by the notably increase in the poultry importation of Western Asian countries, including Saudi Arabia and the United Arab Emirates (FAOSTAT, 2018). Notably, the strain from United Arab Emirates have the same markers (T275 and T280) as the Brazilian sub-clade, reinforcing the hypothesis that this strain descend from strains of Brazilian origin.

## Conclusion

Our findings provide a different paradigm to understand the evolution of IBDV by creating a new timeline to explain the origin and genetic variability of the virus. This hypothesis supposes an early divergence of an IBDV ancestor in two main branches, one corresponding to cIBDV and the other to dIBDV strains, which may have emerged almost at the same time around 1930. It stands out that the cIBDV lineage suffered a notorious diversification giving rise to the vaIBDV and vvIBDV strains while the dIBDV strains maintained more stable along the years, despite some level of local differentiation which is evidenced by the presence of three main sub-populations composed by the European/Argentine/Uruguayan dIBDV strains, the Brazilian dIBDV strains and the Eastern Asian/Canadian dIBDV strains. The presence of sub-population specific residues confirms that the dIBDV diversification is correlated with migration events occurred several years ago. Taken together, our results highlight the complex evolutionary history of IBDV and the importance of new phylodynamic data to unravel and nearly follow the different evolutionary pathways taken by this important poultry pathogen.

## ACKNOWLEDGMENTS

This work was supported by the Instituto Nacional de Investigación Agropecuaria under Project number 319 of the Fondo de Promoción de Tecnología Agropecuaria; Comisión Sectorial de Investigación Científica; Programa de Desarrollo de las Ciencias Básicas; and Agencia Nacional de Investigación e Innovación. We thank Granjas Hepa Ltda. for collaboration.

## CONFLICT OF INTEREST STATEMENT

There is not any conflict of interest in this paper.

## REFERENCES


Alfonso-Morales, A., Martínez-Pérez, O., Dolz, R., Valle, R., Perera, C.L., Bertran, K., Frías, M.T., Majó, N., Ganges, L. & Pérez, L.J. (2013). Spatiotemporal phylogenetic analysis and molecular characterization of infectious bursal disease viruses based on the VP2 hyper-variable region. PLoS ONE, 8, e65999.

Allan, W.H., Faragher, J.T. & Cullen, G.A. (1972). Immunosuppression by the infectious bursal agent in chickens immunised against Newcastle disease. The Veterinary Record, 90, 511–512.

Banda, A., Villegas, P. & El-Attrache, J. (2003). Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. Avian Diseases, 47, 87–95.

Cao, Y.C., Yeung, W.S., Law, M., Bi, Y.Z., Leung, F.C. & Lim, B.L. (1998). Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. Avian Diseases, 42, 340–351.

Chettle, N., Stuart, J.C. & Wyeth, P.J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. The Veterinary Record, 125, 271–272.

Cosgrove, A.S. (1962). An apparently new disease of chickens-avian nephrosis. Avian Diseases, 6, 385–389.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. Journal of Virology, 76, 2393–2402.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. Nature Methods, 9, 772.

de Fraga, A.P., Gräf, T., Coltro, V.P., Ikuta, N., Fonseca, A.S.K., Majó, N. & Lunge, V.N. Phylodynamic analyses of Brazilian antigenic variants of infectious bursal disease virus. Infectious Genetics and Evolution, 73, 159–166.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T., Becht, H. & Teninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. Journal of Virology, 32, 593–605.

Domanska, K., Mato, T., Rivallan, G., Smietanka, K., Minta, Z., de Boisseson, C., Toquin, D., Lomniczi, B., Palya, V. & Eterradossi, N. (2004). Antigenic and genetic diversity of early European isolates of Infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of Infectious bursal disease virus revisited? Archives of Virology, *K*, 465–480.

FAOSTAT. (2018). Food and Agriculture Organization of the United Nations (FAO). http://www.fao.org/faostat/en/#data.

Faragher, J.T., Allan, W.H. & Wyeth, P.J. (1974). Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. The Veterinary Record, 95, 385–388.

Firth, G.A. (1974). Occurrence of an infectious bursal syndrome within an Australian poultry flock. Australian Veterinary Journal, 50, 128–130.

Gao, L., Li, K., Qi, X., Gao, H., Gao, Y., Qin, L., Wang, Y., Shen, N., Kong, X. & Wang, X. (2014). Triplet amino acids located at positions 145/146/147 of the RNA polymerase of very virulent infectious bursal disease virus contribute to viral virulence. Journal of General Virology, 95, 888–897.

Geoghegan, J.L. & Holmes, E.C. (2017). Predicting virus emergence amid evolutionary noise. Open Biology, 7, 170189.

Gordon, J.S. (1996). The chicken story. American Heritage, 47, 53–67.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology, 59, 307–321.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology, 52, 696–704.

He, C.Q., Ma, L.Y., Wang, D., Li, G.R. & Ding, N.Z. (2009). Homologous recombination is apparent in infectious bursal disease virus. Virology, 384, 51–58.

Hernández, M., Tomás, G., Marandino, A., Iraola, G., Maya, L., Mattion, N., Hernández, D., Villegas, P., Banda, A., Panzera, Y. & Pérez, R. (2015). Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. Avian Pathology, 44, 212–221.

Hewson, P. (2007). Origin and development of the British poultry industry: the first hundred years. British Poultry Science, 27, 525–539.

Hon, C.C., Lam, T.Y., Drummond A., Rambaut, A., Lee, Y.F., Yip, C.W., Zeng, F., Lam, P.Y., Ng, P.T. & Leung, F.C. (2006). Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. Journal of Virology, 80, 8503–8509.

Ignjatovic, J. & Sapats, S. (2002). Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. Australian Veterinary Journal, 80, 689–694.

Ikuta, N., El-Attrache, J., Villegas, P., García, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. & Marques, E.K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. Avian Diseases, 45, 297–306.

Ismail, N.M., Saif, Y.M. & Moorhead, P.D. (1988). Lack of pathogenicity of five serotype 2 infectious

bursal disease viruses in chickens. Avian Diseases, 32, 757–759.

Jackwood, D.J. (2012). Molecular epidemiologic evidence of homologous recombination in infectious bursal disease viruses. Avian Diseases, 56, 574–577.

Jackwood, D.J., Saif, Y.M. & Moorhead, P.D. (1985). Immunogenicity and antigenicity of infectious bursal disease virus serotypes I and II in chickens. Avian Diseases, 29, 1184–1194.

Jackwood, D.J., Sommer, S.E. & Knoblich, H. V. (2001). Amino acid comparison of infectious bursal disease viruses placed in the same or different molecular groups by RT/PCR-RFLP. Avian Diseases, 45, 330–339.

Jackwood, D.J. & Sommer, S.E. (1997). Restriction fragment length polymorphism in the VP2 gene of infectious bursal disease viruses. Avian Diseases, 41, 627–637.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal disease viruses from four continents. Virology, 365, 369–375.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Amino acids contributing to antigenic drift in the infectious bursal disease Birnavirus (IBDV). Virology, 409, 33–37.

Jeon, W.-J., Choi, K.-S., Lee, D.-W., Lee, E.-K., Cha, S.-H., Cho, S.-H., Kwon, J.-H., Yoon, Y., Kim, S.-J., Kim, J.-H. & Kwon, H.-J. (2009). Molecular epizootiology of infectious bursal disease (IBD) in Korea. Virus Genes, 39, 342–351.

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, 33, 1870–1874.

Kwon, H.M., Kim, D.K., Hahn, T.W., Han, J.H. & Jackwood, D.J. (2000). Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. Avian Diseases, 44, 691–696.

Le Nouën, C., Rivallan, G., Toquin, D., Darlu, P., Morin, Y., Beven, B., de Boisseson, C., Cazaban, C., Comte, S., Gardin, Y. & Eterradossi, N. (2006). Very virulent infectious bursal disease virus: reduced pathogenicity in a rare natural segment-B-reassorted isolate. Journal of General Virology, 87, 209–216.

Lejal, N., Costa, B.D., Huet, J.C., Delmas, B. & Da Costa, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. Journal of General Virology, 81, 983–992.

Letunic, I. & Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Research, 44, W242–245.

Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodríguez, J.F. & Castón, J.R. (2009). Infectious Bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. Journal of Molecular Biology, 386, 891–901.

Lupini, C., Giovanardi, D., Pesente, P., Bonci, M., Felice, V., Rossi, G., Morandini, E., Cecchinato, M. & Catelli, E. (2016). A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. Avian Pathology, 45, 458–464.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. & Allan, G.M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. Avian Pathology, 9, 395–404.

McIlroy, S.G., Goodall, E.A. & McCracken, R.M. (1989). Economic effects of subclinical infectious bursal disease on broiler production. Avian Pathology, 18, 465–480.

Michel, L.O. & Jackwood, D.J. (2017). Classification of infectious bursal disease virus into genogroups. Archives of Virology, 162, 3661–3670.

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA. pp 1–8.

Minta, Z., Roszkowski, J., Karczewski, W. & Kozaczynski, W. (1985a). Properties of indigenous strains of infectious bursal disease virus. II. Pathogenicity. Bulletin of the Veterinary Institute in Pulawy, 28–29, 67–73.

Minta, Z., Roszkowski, J., Karczewski, W. & Kozaczynski, W. (1985b). Properties of indigenous strains of infectious bursal disease virus. III. Immunosuppressive effect. Bulletin of the Veterinary Institute in Pulawy, 28–29, 73–76.

Müller, H., Scholtissek, C. & Becht, H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. Journal of Virology, 31, 584–589.

Mundt, E., Beyer, J. & Müller, H. (1995). Identification of a novel viral protein in infectious bursal disease virus-infected cells. Journal of General Virology, 76, 437–443.

Ojkic, D., Martin, E., Swinton, J., Binnington, B. & Brash, M. (2007). Genotyping of Canadian field strains of infectious bursal disease virus. Avian Pathology, 36, 427–433.

Pikula, A., Lisowska, A., Jasik, A. & Śmietanka, K. (2018). Identification and assessment of virulence of a natural reassortant of infectious bursal disease virus. Veterinary Research, 49, 89.

Remorini, P., Calderón, M.G., Aguirre, S., Periolo, O., La Torre, J. & Mattion, N. (2006). Characterization of infectious bursal disease viruses from Argentina. Avian Diseases, 50, 245–251.

Rosales, A.G., Villegas, P., Lukert, P.D., Fletcher, O.J. & Brown, J. (1989). Immunosuppressive potential and pathogenicity of a recent isolate of infectious bursal disease virus in commercial broiler chickens. Avian Diseases, 33, 724–728.

Rosenberger, J. K., Cloud S.S., Gelb Jr. J., Odor E., and Dohms J.E. (1985). Sentinel bird survey of Delmarva broiler flocks. In: Proceedings of the 20th National Meeting on Poultry Health and Condemnations, Ocean City, MD. pp. 94-101.

Rosenberger, J.K. & Gelb, J. (1978). Response to several avian respiratory viruses as affected by infectious bursal disease virus. Avian Diseases, 22, 95–105.

Sánchez, A.B., Rodriguez, J.F. & Sanchez, A.B. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. Virology, 262, 190–199.

Sharma, J.M., Dohms, J.E. & Metz, A.L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. Avian Diseases, 33, 112–124.

Shcherbakova, L.O., Lomakin, A.I., Borisov, A. V, Drygin, V. V & Gusev, A.A. (1998). [Comparative analysis of the VP2 variable region of the gene from infectious bursal disease virus isolates]. Molekuliarnaia Genetika, Mikrobiologiia i Virusologiia, 1, 35–40.

Silva, F.M., Vidigal, P.M., Myrrha, L.W., Fietto, J.L., Silva, A.Jr. & Almeida, M.R. (2013). Tracking the molecular epidemiology of Brazilian Infectious bursal disease virus (IBDV) isolates. Infectious, Genetics and Evoution, 13, 18–26.

Soubies, S.M., Courtillon, C., Briand, F.-X., Queguiner-Leroux, M., Courtois, D., Amelot, M., Grousson, K., Morillon, P., Herin, J.-B. & Eterradossi, N. (2017). Identification of a European interserotypic reassortant strain of infectious bursal disease virus. Avian Pathology, 46, 19–27.

Spies, U., Müller, H. & Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. Virus Research, 8, 127–140.

Tomás, G., Hernández, M., Marandino, A., Panzera, Y., Maya, L., Hernández, D., Pereda, A., Banda, A., Villegas, P., Aguirre, S. & Pérez, R. (2012). Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. Journal of Virological Methods, 185, 101–107.

Tomás, G., Hernández, M., Marandino, A., Techera, C., Grecco, S., Hernández, D., Banda, A., Panzera, Y. & Pérez, R. (2017). Development of an RT-qPCR assay for the specific detection of a distinct genetic lineage of the infectious bursal disease virus. Avian Pathology, 46, 150–156.

Tomás, G., Marandino, A., Courtillon, C., Amelot, M., Keita, A., Pikula, A., Hernández, M., Hernández, D., Vagnozzi, A., Panzera, Y., Domańska-Blicharz, K., Eterradossi, N., Pérez, R. & Soubies, S.M. (2019). Antigenicity, pathogenicity and immunosuppressive effect caused by a South American isolate of infectious bursal disease virus belonging to the “distinct” genetic lineage. Avian Pathology, 48, 245–254.

Vakharia, V.N., He, J., Ahamed, B. & Snyder, D.B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. Virus Research, 31, 265–273.

van den Berg, T.P., Gonze, M. & Meulemans, G. (1991). Acute infectious bursal disease in poultry: Isolation and characterisation of a highly virulent strain. Avian Pathology, 20, 133–143.

Vera, F., Craig, M.I., Olivera, V., Rojas, F., König, G., Pereda, A. & Vagnozzi, A. (2015). Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. Archives of Virology, 160, 1909–1921.

Yamazaki, K., Ohta, H., Kawai, T., Yamaguchi, T., Obi, T. & Takase, K. (2017). Characterization of variant infectious bursal disease virus from a broiler farm in Japan using immunized sentinel chickens. Journal of Veterinary Medical Science, 79, 175–183.

Zachar, T., Popowich, S., Goodhope, B., Knezacek, T., Ojkic, D., Willson, P., Ahmed, K.A. & Gomis, S. (2016). A 5-year study of the incidence and economic impact of variant infectious bursal disease viruses on broiler production in Saskatchewan, Canada. Canadian Journal of Veterinary Research, 80, 255–261.

Zhou, J., Liu, F., Tao, S. & Ai, G. (1981). Isolation of pathogen of infectious bursal disease in Beijing area. Chinese Journal of Veterinary Medicine, 8, 25–26.

Table 1- Argentine and Uruguayan dIBDV strains here analyzed.

| Strain name | Origin | Collection Date | Obtained sequence |
|---|---|---|---|
| 1/chicken/ARG/P3/15 | Argentina | 2015 | hvVP2 † |
| 1/chicken/ARG/P4/15 | Argentina | 2015 | hvVP2/pVP1‡ |
| 1/chicken/ARG/P13/15 | Argentina | 2015 | hvVP2 |
| 1/chicken/ARG/P14/15 | Argentina | 2015 | hvVP2 |
| 1/chicken/ARG/P20/15 | Argentina | 2015 | hvVP2 |
| 1/chicken/ARG/P33/15 | Argentina | 2015 | Complete genome |
| 1/chicken/ARG/P46/15 | Argentina | 2015 | hvVP2/pVP1 |
| 1/chicken/ARG/P59/16 | Argentina | 2016 | hvVP2 |
| 1/chicken/ARG/P60/16 | Argentina | 2016 | hvVP2 |
| 1/chicken/ARG/P79/16 | Argentina | 2016 | hvVP2/pVP1 |
| 1/chicken/ARG/P81/16 | Argentina | 2016 | hvVP2/pVP1 |
| 1/chicken/ARG/P85/16 | Argentina | 2016 | hvVP2/pVP1 |
| 1/chicken/ARG/P97/16 | Argentina | 2016 | hvVP2 |
| M04/09 | Uruguay | 2009 | Complete genome |
| 421101 | Uruguay | 2011 | Complete genome |
| 1/chicken/URY/4202/11 | Uruguay | 2011 | hvVP2 |
| 1/chicken/URY/4203/11 | Uruguay | 2011 | hvVP2/pVP1 |
| 221201 | Uruguay | 2012 | Complete genome |
| 1/chicken/URY/2701/12 | Uruguay | 2012 | Complete genome |
| 1/chicken/URY/3401/12 | Uruguay | 2012 | hvVP2/pVP1 |
| 1/chicken/URY/3501/12 | Uruguay | 2012 | hvVP2/pVP1 |
| 1/chicken/URY/1403/14 | Uruguay | 2014 | hvVP2 |
| 1/chicken/URY/1302/16 | Uruguay | 2016 | Complete genome |
| 1/chicken/URY/1305/16 | Uruguay | 2016 | hvVP2/pVP1 |

† hvVP2: hypervariable region of VP2.

‡ pVP1: partial VP1 gene.

Table 2- Amino acid markers of the dIBDV strains found in VP5, polyprotein and VP1.

| | | | VP5 | | | Polyprotein | | | | | | | | | | | | VP1 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | VP2 | | | | | | | | VP4 | | VP3 | | | | | | | | |
| | | | 40 | 135 | 137 | 5 | 124 | 222† | 245 | 272† | 289 | 290 | 296 | 680 | 711 | 777 | 922 | 56 | 57 | 145 | 243 | 305 | 651 | 859 |
| cIBDV‡ | | | S | H | R | Q | I | P | E | I | L | M | I | C | V | N | L | E | N | N | F | S | H | T |
| vaIBDV‡ | | | S | H | R | Q | I | T/Q | E | I | L | M | I | C | V | N | L | E | N | N | F | S | H | T |
| vvIBDV‡ | | | S | H | R | Q | I | A | E | I | L | M | I | Y | V | N | L | E | N | T | F | S | H | T |
| dIBDV | ARGENTINA & URUGUAY | M04/09 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | I |
| | | 421101 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | T |
| | | 221201 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | T |
| | | 1/chicken/URY/2701/12 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | T |
| | | 1/chicken/URY/1302/16 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | T |
| | | 1/chicken/ARG/P33/15 | P | R | R | P | V | S | G | T | P | I | F | W | I | S | I | D | S | S | P | S | Q | I |
| | POLAND | 80GA | — | — | — | — | — | S | G | T | P | I | F | — | — | — | — | — | — | N | P | — | — | — |
| | | 78/GSz | — | — | — | — | — | S | G | T | P | I | F | — | — | — | — | — | — | N | P | — | — | — |
| | BRAZIL | Candioto | S | R | C | — | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | Giacomini | S | R | C | — | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | Brandeleiro | S | R | C | — | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | Prezotto | S | R | C | — | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | MG4 | S | R | C | Q | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | SP9 | S | R | C | Q | V | S | E | T | P | I | F | — | — | — | — | — | — | — | — | — | — | — |
| | | MG8 | S | R | C | Q | V | S | E | T | P | I | F | — | — | — | — | D | S | D | F | G | H | S |
| | | MG1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | D | S | D | F | G | H | S |
| | | SC11 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | D | S | D | F | G | H | S |

Black background residues: strong markers found in all the dIBDV strains (collected from a wide range of years and geographic regions); dark gray background residues: potential dIBDV markers, consistently found in the few number of available strains; light gray background residues: sub-population specific markers.

† markers also detected in some non-dIBDV strains (S222 in the cIBDV vaccine strains Lukert and 228E; T272 in the G7 strains).

‡ most frequent amino acid residues.

Table 3- Year of origin estimated for the most recent common ancestor of the cIBDV, vaIBDV, vvIBDV and dIBDV strains.

| | Year | 95% HPD† |
|---|---|---|
| cIBDV | 1927 | 1901 – 1949 |
| vaIBDV | 1949 | 1925 – 1971 |
| vvIBDV | 1967 | 1957 – 1977 |
| dIBDV | 1931 | 1898 – 1963 |

† 95% HPD: 95% High Probability Density.

## FIGURE LEGENDS

Figure 1- Maximum likelihood based phylogenetic tree of IBDV using a 356-nt fragment of the hvVP2 region. The GTR+I+G nucleotide substitution model was used. Each genogroup and the serotype 2 strains are indicated with different colours. Nodes with aLRT support values above 0.75 are indicated with grey circles.

Figure 2- Maximum likelihood based phylogenetic tree of IBDV using a 533-nt fragment of the VP1 gene. The nucleotide substitution model was GTR+I+G. The cIBDV (G1), vaIBDV (G2) and G6 strains form a clade indicated with a dotted rectangle. Nodes with aLRT support values above 0.75 are indicated with grey circles.

Figure 3- Maximum likelihood based phylogenetic trees using a 3070-nt fragment of the IBDV genome segment A (left) and a 2634-nt fragment corresponding to the IBDV genome segment B (right).
In both cases the GTR+I+G nucleotide substitution model was used. Each genogroup and serotype 2 strains are indicated with colours according to the code detailed in the bottom of the figure. The monophyletic group composed by cIBDV (G1) and vaIBDV (G2) strains is indicated with a dotted rectangle.

Figure 4- Maximum Clade Credibility Tree (MCCT) of the dIBDV (G4) strains using the hvVP2 region. To the upper left is a graph representing the root location state posterior probabilities. The branches and nodes are coloured according to the most probable location state following the code used in the graph. The tMRCA is indicated for the basal and other relevant nodes.

Figure 5- World map indicating the migration events estimated for the dIBDV strains. Coloured dots indicate the different countries included in the analysis. Countries that were grouped in the same location state are indicated with coloured ovals. Arrows' colour and thickness represent the time and BF support, respectively, of each migration event.

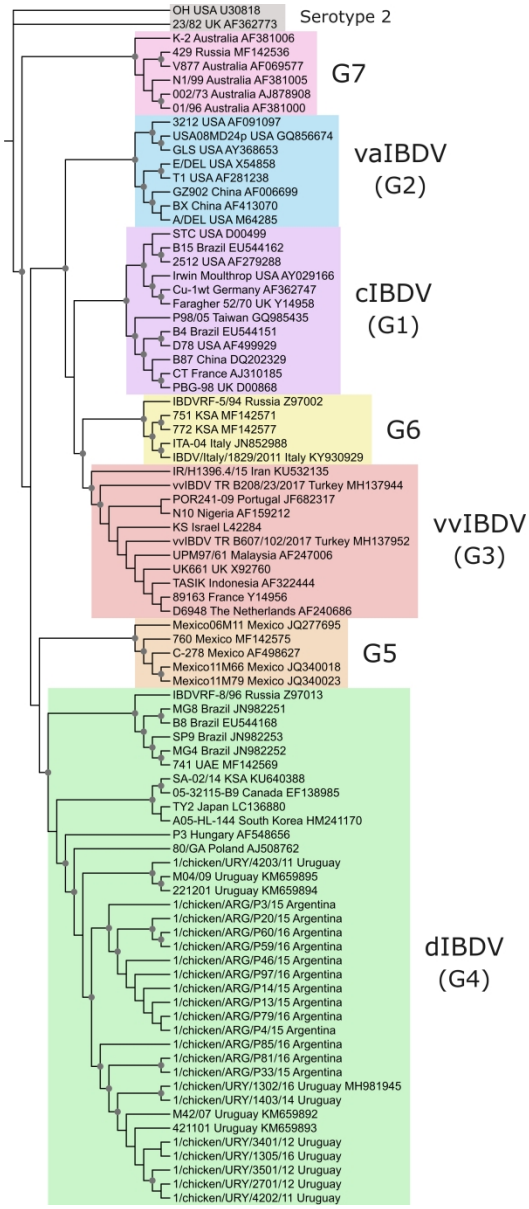


Figure 1- Maximum likelihood based phylogenetic tree of IBDV using a 356-nt fragment of the hvVP2 region. The GTR+I+G nucleotide substitution model was used. Each genogroup and the serotype 2 strains are indicated with different colours. Nodes with aLRT support values above 0.75 are indicated with grey circles.

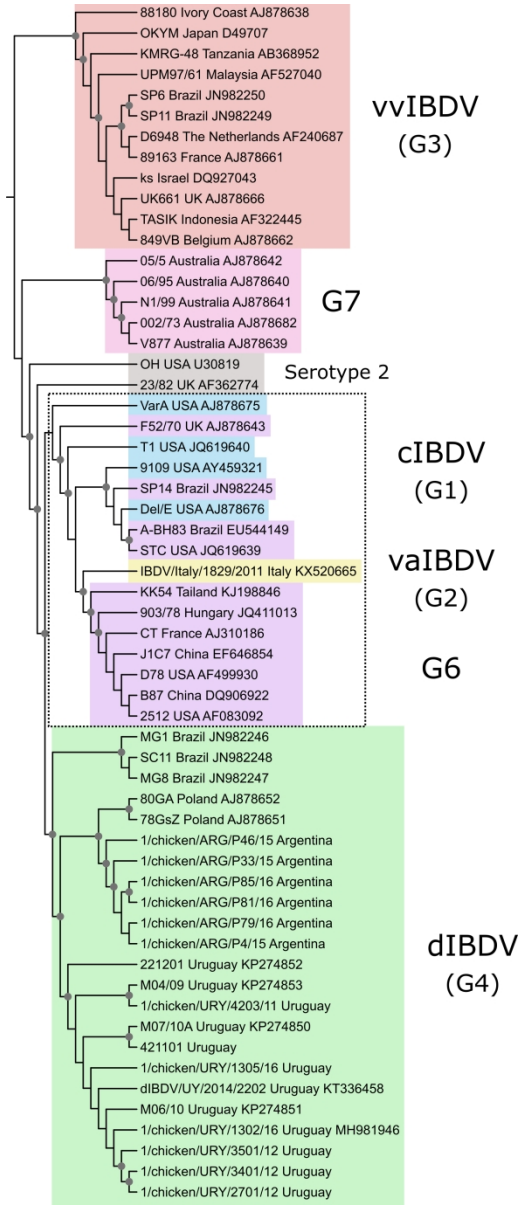


Figure 2: Maximum likelihood based phylogenetic tree of IBDV using a 533-nt fragment of the VP1 gene. The nucleotide substitution model was GTR+I+G. The cIBDV (G1), vaIBDV (G2) and G6 strains form a clade indicated with a dotted rectangle. Nodes with aLRT support values above 0.75 are indicated with grey circles.

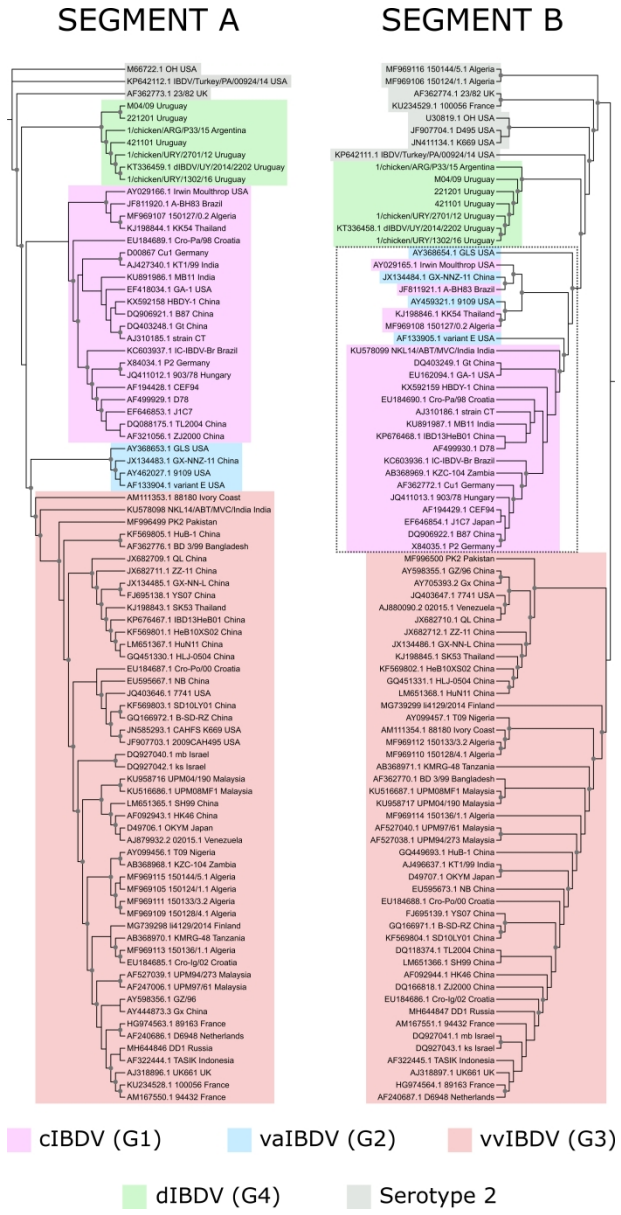


Figure 3: Maximum likelihood based phylogenetic trees using a 3070-nt fragment of the IBDV genome segment A (left) and a 2634-nt fragment corresponding to the IBDV genome segment B (right). In both cases the GTR+I+G nucleotide substitution model was used. Each genogroup and serotype 2 strains are indicated with colours according to the code detailed in the bottom of the figure. The monophyletic group composed by cIBDV (G1) and vaIBDV (G2) strains is indicated with a dotted rectangle.

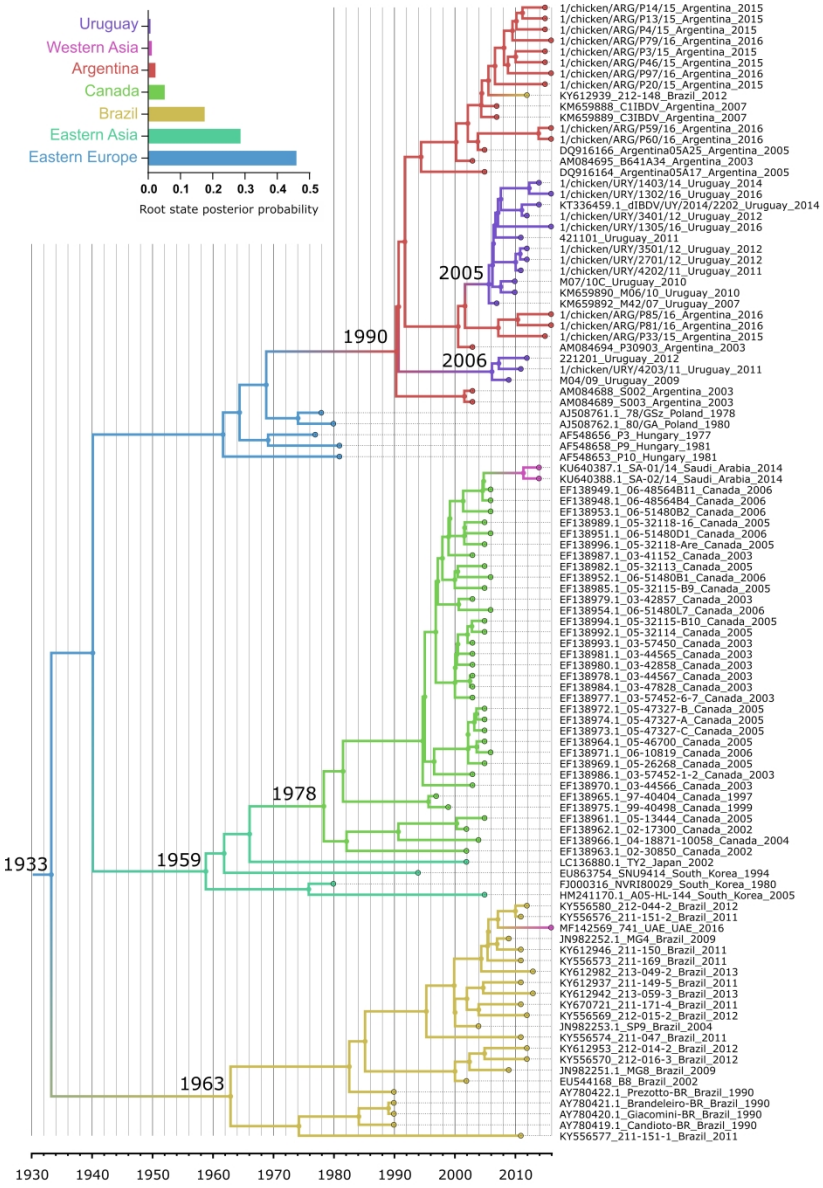


Figure 4: Maximum Clade Credibility Tree (MCCT) of the dIBDV (G4) strains using the hvVP2 region. To the upper left is a graph representing the root location state posterior probabilities. The branches and nodes are coloured according to the most probable location state following the code used in the graph. The tMRCA is indicated for the basal and other relevant nodes.

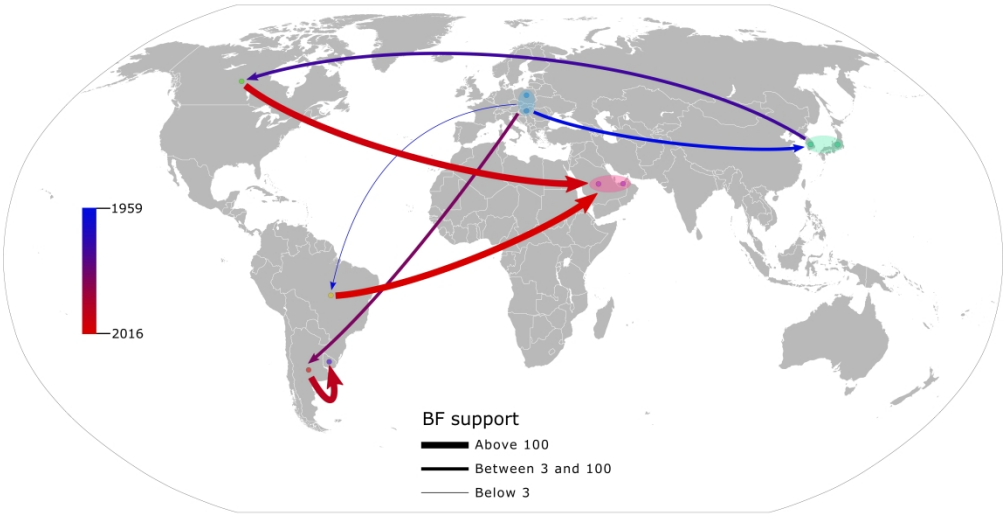


Figure 5: World map indicating the migration events estimated for the dIBDV strains. Coloured dots indicate the different countries included in the analysis. Countries that were grouped in the same location state are indicated with coloured ovals. Arrows' colour and thickness represent the time and BF support, respectively, of each migration event.

Supporting information 1- Primers and cycling conditions used for the complete genome amplification of the genogroup 4 strains.

| Primer name | Sequence (5'→ 3') | Amplicon size (nt) | Genome Segment | Reference |
|---|---|---|---|---|
| F36 | GGGACAGGCCGTCAAGGC | 1192 | A | [1] |
| P4 | GCGACCGTAACGACAGATCC | | | [2] |
| A2f | AGATGTCATGGTCAGCAAGTGGGA | 1262 | A | This study |
| A2r2 | GGGTGGAAGGTATGGWAGGTTGAGG | | | This study |
| A3f | TCAACGCCTATGGCGAGATTGAGA | 1092 | A | This study |
| A3r | GCGAACGGATCCAATTTGGGATGT | | | This study |
| MBF | AGTGGCTCCTCTTCTTGATGATTCTAC | 1104 | B | [3] |
| B1r | TTGGACATCACGGGCCAGGT | | | This study |
| B2f | ATCAACCTCAAGTCATCAAGTGG | 1162 | B | This study |
| B2r | TGTTCAGGAGTGGGTAGTTC | | | This study |
| B3f | ATCTTGGGATCTATGTGCCG | 946 | B | This study |
| B3r | GGGTCTGGGGTTAGTGTC | | | This study |

| Stage | Initial denaturation | Denaturation | Annealing | Extension | Final extension |
|---|---|---|---|---|---|
| Time | 5 min | 30 sec | 30 sec | 1:20 min | 5 min |
| Temperature (°C) | 95 | 95 | 60 | 72 | 72 |
| Cycles | 1 | 35 | | | 1 |

References

[1] Tomás et al., 2012
[2] Liu et al., 1998
[3] Hernández et al., 2011

Supporting information 2 - Recombinant segment A and B sequences detected by the RDP4 software.

| Genome segment | Putative recombinant (accession number) | Putative major parent (accession number) | Putative minor parent (accession number) | Detecting programs | Putative breakepoints | Probability (MC Corrected) |
|---|---|---|---|---|---|---|
| A | KK1 (AF165150) | K310 (AF165149) | 100056 (KU234528) | 7 | 672 - 1789 | $7{,}7 \times 10^{-23}$ |
| | PY12 (KX223749) | THI14/ABT/MVC/India (KU578104) | K310 (AF165149) | 7 | 1436 | $2{,}8 \times 10^{-23}$ |
| | KSH (AF165151) | K310 (AF165149) | 100056 (KU234528) | 7 | 672 - 1789 | $1{,}4 \times 10^{-20}$ |
| | RPM14/ABT/MVC/India (KU578102) | IBDV80/Kaduna.NG/2011 (JX424079) | MB11 (KU891986) | 7 | 2182 - 2738 | $1{,}9 \times 10^{-10}$ |
| | MDI14/ABT/MVC/India (KU558699) | HYA15/ABT/MVC/India (KY020408) | India/BGE15/ABT/MVC/2015 (KT870148) | 4 | 338 - 2017 | $7{,}0 \times 10^{-9}$ |
| | HYA15/ABT/MVC/India (KY020408) | CAHFS-785-SESW (JF907702) | IC-IBDV-Br (KC603937) | 7 | 1386 - 1976 | $5{,}2 \times 10^{-8}$ |
| | EDE14/ABT/MVC/India (KU558697) | HYA15/ABT/MVC/India (KY020408) | THI14/ABT/MVC/India (KU578104) | 4 | 325 - 1311 | $4{,}1 \times 10^{-6}$ |
| | THI14/ABT/MVC/India (KU578104) | UPM08MF1 (KU516686) | BD3/99 (AF362776) | 4 | 369 - 1426 | $6{,}4 \times 10^{-4}$ |
| B | HN04 (KC109815) | Soroa (EF688065) | OKYM (D49707) | 5 | 436 - 2088 | $4{,}8 \times 10^{-61}$ |
| | MDI14/ABT/MVC/India (KU558700) | VCN14/ABT/MVC/India (KU578101) | PY12 (KX223750) | 7 | 932 - 1728 | $4{,}7 \times 10^{-38}$ |
| | HYA15/ABT/MVC/India (KY020409) | Ts (AF203880) | SH99 (LM651366) | 7 | 542 - 1039 | $9{,}3 \times 10^{-22}$ |
| | VCN14/ABT/MVC/India (KU578101) | Ts (AF203880) | PY12 (KX223750) | 7 | 2159 - 2584 | $1{,}4 \times 10^{-22}$ |
| | RPM14/ABT/MVC/India (KU578103) | VCN14/ABT/MVC/India (KU578101) | KT1/99 (AJ496637) | 7 | 1212 - 1526 | $2{,}9 \times 10^{-21}$ |
| | THI14/ABT/MVC/India (KU578105) | VCN14/ABT/MVC/India (KU578101) | PY12 (KX223750) | 7 | 932 - 1570 | $6{,}5 \times 10^{-19}$ |
| | PY12 (KX223750) | Ts (AF203880) | HuB-1 (GQ449693) | 7 | 1970 - 2146 | $1{,}1 \times 10^{-12}$ |
| | CA-K785 (JF907705) | Cu-1wt (AF362748) | 7741 SEGB PROXIMAL (JQ403647) | 4 | 524 - 890 | $4{,}2 \times 10^{-7}$ |

Supporting information 3- Path Sampling (PS) and Stepping Stone Sampling (SSS) results

Results for the cIBDV, vaIBDV, vvIBDV and dIBDV dataset:

| Model_combination | PS | SSS |
|---|---|---|
| ST_CON | -5704.9397 | -5707.2310 |
| ST_EXP | -5695.8961 | -5697.6983 |
| ST_BSP | -5678.0107 | -5679.1045 |
| UL_CON | -5687.1749 | -5689.3008 |
| UL_EXP | -5669.5453 | -5672.4014 |
| **UL_BSP** | **-5665.1117** | **-5668.9324** |
| UE_CON | -5678.6735 | -5682.4613 |
| UE_EXP | -5671.8421 | -5673.9026 |
| UE_BSP | -5670.3124 | -5672.6360 |

Results for the dIBDV dataset:

| Model_combination | PS | SSS |
|---|---|---|
| ST_CON | -3281.9427 | -3282.5176 |
| ST_EXP | -3276.2670 | -3278.8701 |
| ST_BSP | -3275.5633 | -3278.6602 |
| UL_CON | -3275.7529 | -3276.5377 |
| UL_EXP | -3275.0905 | -3275.6862 |
| UL_BSP | -3266.0810 | -3269.2277 |
| UE_CON | -3277.0515 | -3278.1063 |
| **UE_EXP** | **-3265.9373** | **-3267.7965** |
| UE_BSP | -3273.7567 | -3274.5712 |

AVIAN PATHOLOGY
https://doi.org/10.1080/03079457.2019.1572867

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE



# Antigenicity, pathogenicity and immunosuppressive effect caused by a South American isolate of infectious bursal disease virus belonging to the "distinct" genetic lineage

Gonzalo Tomás[a], Ana Marandino[a], Céline Courtillon[b], Michel Amelot[b], Alassane Keita[b], Anna Pikula[c], Martín Hernández[a], Diego Hernández[a], Ariel Vagnozzi[d], Yanina Panzera[a], Katarzyna Domańska-Blicharz[c], Nicolas Eterradossi[b], Ruben Pérez[a] and Sébastien Mathieu Soubies[b]

[a]Sección Genética Evolutiva, Departamento de Biología Animal, Facultad de Ciencias, Instituto de Biología, Universidad de la República Montevideo, Uruguay; [b]Avian and Rabbit Virology Immunology and Parasitology Unit (VIPAC), French Agency for Food, Environmental and Occupational Health Safety (ANSES) Ploufragan, France; [c]Department of Poultry Diseases, National Veterinary Research Institute Pulawy, Poland; [d]Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria Buenos Aires, Argentina

**ABSTRACT**

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious immunosuppressive disease affecting young chickens. The recently described "distinct IBDV" (dIBDV) genetic lineage encompasses a group of worldwide distributed strains that share conserved genetic characteristics in both genome segments making them unique within IBDV strains. Phenotypic characterization of these strains is scarce and limited to Asiatic and European strains collected more than 15 years ago. The present study aimed to assess the complete and comprehensive phenotypic characterization of a recently collected South American dIBDV strain (1/chicken/URY/1302/16). Genetic analyses of both partial genome segments confirmed that this strain belongs to the dIBDV genetic lineage and that it is not a reassortant. Antigenic analysis with monoclonal antibodies indicated that this strain has a particular antigenic profile, similar to that obtained in a dIBDV strain from Europe (80/GA), which differs from those previously found in the traditional classic, variant and very virulent strains. Chickens infected with the South American dIBDV strain showed subclinical infections but had a marked bursal atrophy. Further analysis using Newcastle disease virus-immunized chickens, previously infected with the South American and European dIBDV strains, demonstrated their severe immunosuppressive effect. These results indicate that dIBDV strains currently circulating in South America can severely impair the immune system of chickens, consequently affecting the local poultry industry. Our study provides new insights into the characteristics and variability of this global genetic lineage and is valuable to determine whether specific control measures are required for the dIBDV lineage.

**Research Highlights**

- A South American strain of the dIBDV lineage was phenotypically characterized.
- The strain produced subclinical infections with a marked bursal atrophy.
- Infected chickens were severely immunosuppressed.
- The dIBDV strains are antigenically divergent from other IBDV lineages.

**ARTICLE HISTORY**
Received 31 October 2018
Accepted 17 January 2019

**KEYWORDS**
Infectious bursal disease virus; "distinct IBDV"; South America; pathogenicity; immunosuppression; antigenicity

## Introduction

Infectious bursal disease (IBD) is a highly contagious viral infection that causes severe economic losses in the global poultry industry. Its aetiologic agent is infectious bursal disease virus (IBDV), which belongs to the genus *Avibirnavirus* within the family *Birnaviridae* (Dobos *et al.*, 1979). The virus performs a lytic replication cycle in developing B lymphocytes of the bursa of Fabricius and other lymphoid and non-lymphoid tissues (Burkhardt & Müller, 1987; Abdul *et al.*, 2013). The resulting B lymphocyte depletion affects the immune system of young chicks and leads to an increased susceptibility to other infectious diseases, and poor vaccine responses (Allan *et al.*, 1972; Faragher *et al.*, 1974; Rosenberger & Gelb, 1978).

IBDV has a non-enveloped icosahedral capsid containing a double-stranded RNA genome with two segments, designated A and B (Müller *et al.*, 1979). Segment A (3.3 kbp) encodes a polyprotein that is self-cleaved to release the capsid protein VP2, the viral protease VP4, the ribonucleoprotein VP3 and four peptides necessary for viral entry and assembly

**CONTACT** Ruben Pérez rperez@fcien.edu.uy Sección Genética Evolutiva, Departamento de Biología Animal, Facultad de Ciencias, Instituto de Biología, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay

Supplemental data for this article can be accessed at https://doi.org/10.1080/03079457.2019.1572867

(Sánchez & Rodriguez, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002; Chevalier *et al.*, 2005; Luque *et al.*, 2009). A 5′-terminal overlapping open reading frame encodes the non-structural protein VP5 (Mundt *et al.*, 1995). Segment B (2.9 kbp) encodes the RNA-dependent RNA polymerase VP1 (Spies *et al.*, 1987).

Two viral serotypes (1 and 2) are currently known but only the serotype 1 causes disease in chickens (McFerran *et al.*, 1980; Ismail *et al.*, 1988). According to antigenic and pathogenic characteristics, serotype 1 was traditionally subdivided into classic, antigenic variant and very virulent strains. The classic strains were first detected in North America, causing high morbidity and mortality before specific vaccines were developed to partially control the disease (Cosgrove, 1962; Snedeker *et al.*, 1967; Edgar & Cho, 1976). Classic attenuated strains cause less severe effects than typical "classic virulent" strains (Giambrone & Clay, 1986; Mazariegos *et al.*, 1990), and have been used as live attenuated vaccines since the mid-1970s. Variants with antigenic changes in the neutralizing epitopes of VP2 were detected in North America in the early 1980s (Rosenberger *et al.*, 1985). These antigenic variants were able to evade the maternal immunity induced by classic-type vaccines and produced subclinical infections with marked immunosuppression (Rosales *et al.*, 1989; Sharma *et al.*, 1989). Very virulent strains were first reported in Europe in the late 1980s and caused extremely severe clinical signs, gross and microscopic lesions, and immunosuppression with unusual high mortality (Chettle *et al.*, 1989; van den Berg *et al.*, 1991). The very virulent strains are antigenically similar to the classic strains, but they can break through high levels of maternally-derived antibodies (van den Berg *et al.*, 1996).

IBDV strains can be also distinguished by the affinity profile of different panels of monoclonal antibodies (mAbs) and by sequence analysis of the partial viral genome (Snyder *et al.*, 1992; Eterradossi *et al.*, 1997; Le Nouën *et al.*, 2005). Phylogenetic analysis using sequences corresponding to the hyper-variable region of VP2 (hvVP2 region) can be used to distinguish the genetic lineages corresponding to most antigenically significant strains and also identify new lineages that usually comprise less phenotypically characterized strains (Ignjatovic & Sapats, 2002; Hernández *et al.*, 2015; Lupini *et al.*, 2016). A recent comprehensive analysis proposed a new classification scheme of IBDV strains into genogroups, which is expected to improve the current somewhat confusing nomenclature (Michel & Jackwood, 2017; Jackwood *et al.*, 2018). According to this new scheme, the classic, antigenic variant and very virulent strains correspond to Genogroup 1, Genogroup 2 and Genogroup 3, respectively.

The recently described "distinct IBDV" (dIBDV) lineage is a major lineage that is highly genetically divergent from traditional IBDV subtypes (Hernández *et al.*, 2015). This corresponds to the Genogroup 4 according to the new proposed nomenclature. This lineage includes strains collected in the last four decades from several countries of South and North America, Asia and Europe (Shcherbakova *et al.*, 1998; Kwon *et al.*, 2000; Ikuta *et al.*, 2001; Jackwood *et al.*, 2001; Domanska *et al.*, 2004; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007; Jeon *et al.*, 2009; Hernández *et al.*, 2015; Vera *et al.*, 2015; Michel & Jackwood, 2017; Yamazaki *et al.*, 2017). In the past, dIBDV constituted the majority of IBDV strains identified in Brazil and Canada (Ikuta *et al.*, 2001; Ojkic *et al.*, 2007). More recent epidemiological reports have indicated that dIBDV strains circulate at high frequency in poultry farms from Argentina and Uruguay (Hernández *et al.*, 2015; Vera *et al.*, 2015).

Five Hungarian and Polish dIBDV strains collected from 1977–1981 were analysed using a panel of eight mAbs in an AC-ELISA assay and showed unique antigenic profiles (Domanska *et al.*, 2004). These Polish strains, besides two dIBDV strains collected in 1994 and 2002 from Asia, caused subclinical infections with bursal atrophy and underlying immunosuppression (Minta *et al.*, 1985a, 1985b; Jeon *et al.*, 2009; Yamazaki *et al.*, 2017). All these studies were performed with European and Asiatic strains collected many years ago.

The present study assesses a complete and comprehensive genetic and phenotypic characterization of a currently circulating dIBDV strain from South America in order to provide updated insights into the characteristics and variability of this global genetic lineage.

## Materials and methods

### Ethic statements

All bird experimental infections were conducted in agreement with national regulations on animal welfare and after approval of the protocols by the host laboratory ethical committee (permit number APAFIS#4945-20 16041316546318 v6, ethical Committee registered at the national level under no. C2EA-16).

### Virus strains

The dIBDV strain 1/chicken/URY/1302/16 was collected in South America (Uruguay) in 2016 from an outbreak occurring in a single poultry farm. Bursae of 25-day-old broilers exhibiting mild respiratory clinical signs were obtained and stored at −80°C. The absence of other common viral pathogens (infectious bronchitis virus, chicken anaemia virus and avian reovirus) was confirmed by RT–PCR or RT-qPCR (Xie *et al.*, 1997; Callison *et al.*, 2006; Techera *et al.*, 2019).

The dIBDV strain 80/GA was isolated in 1980 in Europe (Poland) and was included for comparative purposes. The genetic, antigenic and pathogenic characteristics of this strain, including its immunosuppressive effect, have been already published (Minta *et al.*, 1985a, 1985b; Domanska *et al.*, 2004).

Reference strains F52/70 (classic virulent), 89163 (very virulent) and 94432 (atypical very virulent) were used for inter-strain comparison and as controls (Bygrave & Faragher, 1970; Eterradossi *et al.*, 1992; Eterradossi *et al.*, 1998). NDV strain Hertz/33 was used for challenge in the immunosuppressive assay.

### Genetic characterization of the South American dIBDV strain

A specific qPCR assay was used to identify the South American strain as belonging to the dIBDV lineage (Tomás *et al.*, 2017). Further genetic characterization was carried out through partial VP2 and VP1 analyses. Total RNA was extracted directly from infected bursae using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA). Reverse transcription was carried out using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Thermo Scientific, Hanover, MD, USA) with random hexamer primers. A 492-bp fragment within the hvVP2 region and a 594-bp fragment corresponding to a partial sequence of the VP1 gene were amplified. All amplifications were carried out using an Arktik Thermal Cycler (Thermo Scientific) with previously published primers and cycling conditions (Hernández *et al.*, 2006). PCR products were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research) and submitted for bidirectional sequencing (Macrogen Inc, Seoul, South Korea). Sequences were assembled and edited with the Lasergene SeqMan Pro software 15.0.1 (DNASTAR). Two datasets corresponding to the partial VP2 and VP1 coding sequences were generated including the South American strain and representatives of other previously described genetic lineages. Sequence alignments were performed using the MUSCLE algorithm implemented in MEGA 7.0 software (Kumar *et al.*, 2016). The hvVP2 alignment encompassed a 374-bp region of segment A, and the VP1 alignment corresponds to a 543-bp region of segment B. Best-fit nucleotide substitution models were selected under the Akaike information criterion using jModelTest2 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). Maximum-likelihood trees, with an approximate likelihood ratio test (aLRT) for internal nodes support, were inferred using the PhyML 3.0 software (Guindon *et al.*, 2010). Phylogenetic trees were visualized and edited with the TreeGraph2 software and the iTOL v4 online tool (Stöver & Müller, 2010; Letunic & Bork, 2016).

### Virus isolation and titration

Bursae of Fabricius from birds infected with the South American strain were homogenized and treated with 1,1,2-trichlorotrifluoroethane for virus extraction as previously described (Eterradossi *et al.*, 1992). The strain was reinoculated into five specific pathogen-free (SPF) chickens maintained in confined facilities (ANSES, Ploufragan, France). Bursae from the inoculated chickens were harvested 4 days post infection and used to prepare primary viral stocks.

Remaining strains were obtained from viral stocks maintained in facilities of the OIE reference laboratory Ploufragan, France. Virus titration was carried out using primary bursal cell cultures as previously described (Soubies *et al.*, 2018) for the pathogenicity assay, and SPF embryonated chicken eggs for the immunosuppression assay. In both procedures, 10-fold serial dilutions were used as inoculum with eight replicates each. Nine- to eleven-day-old embryonated chicken eggs were used and inoculated by the chorioallantoic membrane (CAM) route. Titration of NDV strain Herts/33 was performed as described for IBDV using intra-allantoic inoculation of 9-day-old embryonated chicken eggs. Titres were calculated by the Reed & Muench method and were expressed as tissue culture infectious dose 50% ($TCID_{50}$) and embryo infectious dose 50% ($EID_{50}$), respectively (Reed & Muench, 1938).

### Antigenic characterization

The antigenic profile of the South American strain was determined by using a previously described antigen-capture ELISA (AC-ELISA) (Eterradossi *et al.*, 1997). Briefly, IBDV antigens were captured in standardized amounts using an anti-IBDV chicken polyclonal serum and detected with a panel of eight IBDV-specific neutralizing mAbs. Binding of the mAbs to the virus particle is expressed as the percentage of the binding of a polyclonal reference reagent (percentage and binding are positively correlated). European dIBDV strain 80/GA was included in the analysis for comparative purposes. Strains F52/70 and 94432 were used as references for classic virulent and atypical very virulent antigenic profiles, respectively.

### Characterization of acute pathogenicity

Acute pathogenicity assessment was performed using 6-week-old SPF White-Leghorn chickens (ANSES) maintained in biosafety level 2 isolators. On the first day, chickens were weighed and divided into three groups of comparable sex and weight. Chickens were identified by coloured rings on their legs. One group of five chickens was mock-inoculated by the intranasal route with sterile culture medium (uninfected controls)

and housed in a positive-pressure filtered air isolator. Two other groups containing 13 and 15 chickens were inoculated with $10^{6.3}$ $TCID_{50}$ (South American isolate) or $10^{8.1}$ $TCID_{50}$ (very virulent 89163), respectively. Clinical signs were recorded daily for 10 days using a symptomatic index ranging from 0 (no clinical sign) to 3 (severely affected or dead bird) (Le Nouën *et al.*, 2012). Five birds belonging to both virus-inoculated groups were euthanatized at 4 days post infection (dpi) to determine gross lesions and record body and bursa weights. The same procedure was performed at 21 dpi with the surviving birds from the infected and uninfected groups. Additionally, serum samples were taken at 21 dpi and screened for antibodies against a panel of avian viruses that included IBDV, avian influenza virus, infectious laryngotracheitis virus, avian adenovirus CELO, avian metapneumovirus, infectious bronchitis virus, chicken anaemia virus and avian reovirus as previously described (Soubies *et al.*, 2017). Bursa to body weight (BBW) ratios were calculated with the formula BBW ratio = bursa weight (g)/body weight (g).

### Evaluation of immunosuppression

Two groups of 13 one-day-old chicks were infected by the eye drop route with $10^2$ $EID_{50}$ of either South American or European dIBDV strains. Two other groups composed of 15 one-day-old chicks were maintained uninfected and used as unvaccinated and vaccinated controls. At day 15, all groups except the unvaccinated control group received one dose of Newcastle disease virus (NDV) live vaccine (Zoetis, Poulvac Hitchner B1) by the eye drop route. At day 30, blood samples were taken from all chicks for NDV serology using haemagglutination inhibition (HI) test. Additionally, three chicks per group were euthanized for necropsic examination. All remaining chicks were then injected intra-muscularly with $2 \times 10^5$ $EID_{50}$ of the NDV virulent strain Herts/33. Clinical signs were followed and mortality was recorded daily for 15 days.

### Statistical analyses

Statistical differences were evaluated using the R version 3.4.4 software (R Core Team, 2014). BBW ratios as well as HI log2 titres were compared using the unpaired *t*-test for two groups of data or the one-way ANOVA followed by Tukey's test when more than two groups of data were compared. Mortality percentage differences were evaluated using Fisher's exact test. Values of $P < 0.05$ were considered statistically significant.

### Accession numbers

The new sequences corresponding to the hvVP2 region and the partial VP1 gene were submitted to the Genbank database with the accession numbers MH981945 and MH981946, respectively.

## Results

### Genetic characterization

The South American (1/chicken/URY/1302/16) and European (80/GA) strains form a dIBDV phylogenetic group (Genogroup 4) based on the hvVP2 nucleotide sequence together with other strains from South America, Europe and Asia (Figure 1). The same clustering is observed with the partial sequence of VP1 region but fewer strains are analysed as segment B is less frequently studied. The remaining observed lineages correspond to classic virulent, classic attenuated, variant and very virulent strains.

The amino acid sequences of the hvVP2 region of the South American and European dIBDV strains are highly conserved and have the signature T272, P289, I290 and F296. Additionally, they have a few common amino acid changes (e.g. similar substitutions at positions 222 and 254) and the only differentiating amino acid is at the position 299 (Supplementary material S1A).

The amino acid sequences of VP1 differ at residue 145 (Supplementary material S1B); European strains have N, which is also present in some classic virulent, classic attenuated and variant strains, whereas the South American dIBDV strains have S. Both dIBDV strains have the P243 VP1 marker.

### Antigenic characterization

The classical virulent strain F52/70 had high reactivity with all eight mAbs tested, while the atypical very virulent strain 94432 lacked reactivity to mAbs 3 and 4, and had a reduced binding affinity with mAbs 5 and 6 (Table 1). South American and European dIBDV strains showed the same antigenic profile. They both lacked binding sites for mAbs 3, 4 and 5, and had a reduced binding affinity with mAbs 1 and 8.

### Characterization of acute pathogenicity

No mortality or clinical signs were observed in both uninfected control group and chickens infected with the South American dIBDV strain (Figure 2(A,B)). The reference very virulent strain 89163 caused 46.7% mortality (7/15) and typical signs of acute infectious bursal disease, including ruffled feathers, diarrhoea and prostration, and had the highest symptomatic index value of 1.67 at 3 dpi.

At 4 dpi, the mean BBW ratios of the very virulent and South American dIBDV strain were 3.76% and 2.45%, respectively; this difference was not statistically significant ($P > 0.05$). At 21 dpi, the uninfected control

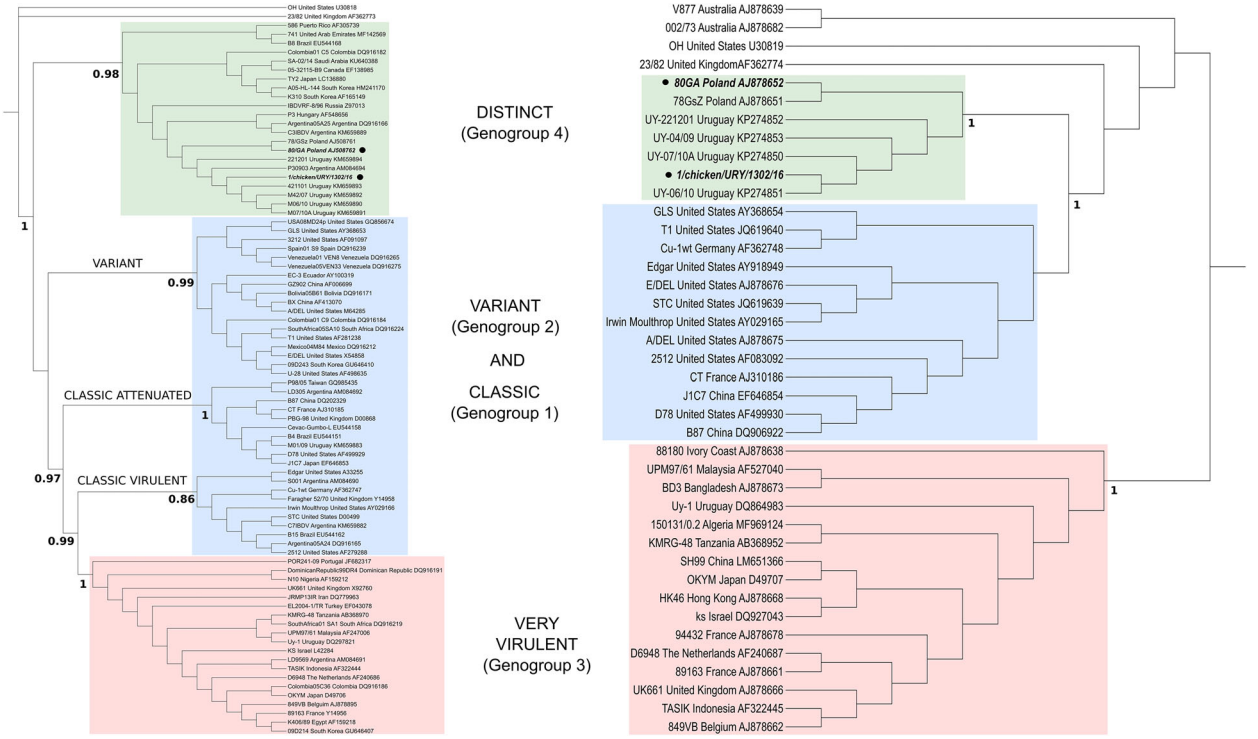


**Figure 1.** Phylogenetic inferences deduced from nucleotide sequences corresponding to the partial hvVP2 region (left) and the partial VP1 gene (right). Main genetic lineages are indicated with shaded boxes. The names of the corresponding genogroups are indicated within brackets. Both trees were inferred using the maximum-likelihood method with the GTR + I+G nucleotide substitution model. Approximate likelihood ratio test support values are shown for relevant nodes. South American and European dIBDV strains analysed in this study are indicated with bold letters and black circles.

had the largest mean value (3.42%), while the very virulent and South American dIBDV strains had significantly lower values (0.48% and 0.85%, respectively) (Figure 2(C)). Serological analyses assessed at the end of the experiment evidenced the presence of antibodies against IBDV and the lack of antibodies against seven other viral pathogens in the infected chickens (data not shown).

## *Evaluation of immunosuppression*

The absence of clinical signs combined with the marked bursal atrophy at 21 dpi in chicks infected with the South American dIBDV strain prompted us to specifically evaluate the immunosppressive properties of this virus, in comparison with the European dIBDV strain 80/GA (Minta *et al.*, 1985b). Immunization with NDV elicited high titres of neutralizing antibodies in the control group (mean value: 5.3 log2) in comparison with the unvaccinated group (mean titre: <2.0 log2) (Figure 3(A)). These titres were significantly lower ($P < 0.05$) in groups inoculated with the South American (mean value: 2.5 log2) or the European virus (mean value: 2.6 log2), with only two out of 10 birds (South American strain) and five out of 10 birds (European strain) exhibiting an antibody response, respectively.

Birds belonging to the vaccinated control group survived the challenge with the NDV virulent strain Herts/33 and lacked any clinical sign of Newcastle disease (Figure 3(B)). All unvaccinated birds succumbed to the challenge with the NDV virulent strain Herts/33. The cumulated mortalities of the dIBDV strains, 70% (7/10) for the South American strain and 44.4% (4/9) for the European strain, were statistically significantly different compared to the vaccinated control ($P < 0.05$). Collectively, these data show that the South American dIBDV strain 1/chicken/URY/1302/16

**Table 1.** Antigenic characterization of the Uruguayan dIBDV strain by AC-ELISA. Light grey = reduced binding; black grey = lack of binding.

| | % AC-ELISA reactivity[a] of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| IBDV strain | Mab 1 | Mab 3 | Mab 4 | Mab 5 | Mab 6 | Mab 7 | Mab 8 | Mab 9 |
| F52/70[b] | 59 | 58 | 70 | 48 | 100 | 73 | 109 | 78 |
| 94432[c] | 62 | 5 | −1 | 25 | 18 | 95 | 92 | 44 |
| 80/GA[c] | 32 | −1 | 0 | −1 | 101 | 96 | 23 | 91 |
| 1/chicken/URY/1302/16[b] | 26 | −1 | 0 | 0 | 105 | 90 | 26 | 73 |

[a]Calculated according to Eterradossi *et al.* (1997).
[b]Mean percentage of two AC-ELISA assays.
[c]Percentage of one AC-ELISA assay.

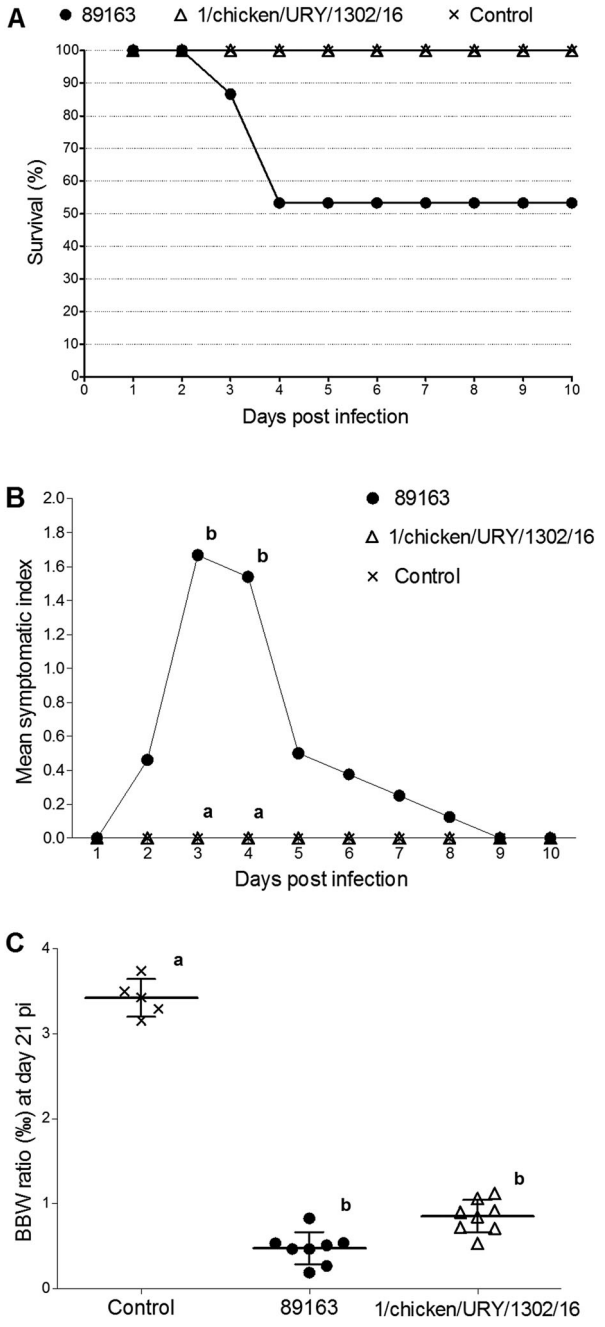


**Figure 2.** Pathogenicity of the South American dIBDV strain compared to the European very virulent strain 89163. (A) Survival rate of 6-week-old SPF chickens infected intranasally with each strain or mock-inoculated, during a 10-day period post inoculation. (B) Mean symptomatic indexes of chickens recorded within 10 days post infection. (C) Bursa to body weight (BBW) ratios calculated 21 days post infection. Statistically significant differences ($P < 0.05$) between selected groups are indicated with different lowercase letters.

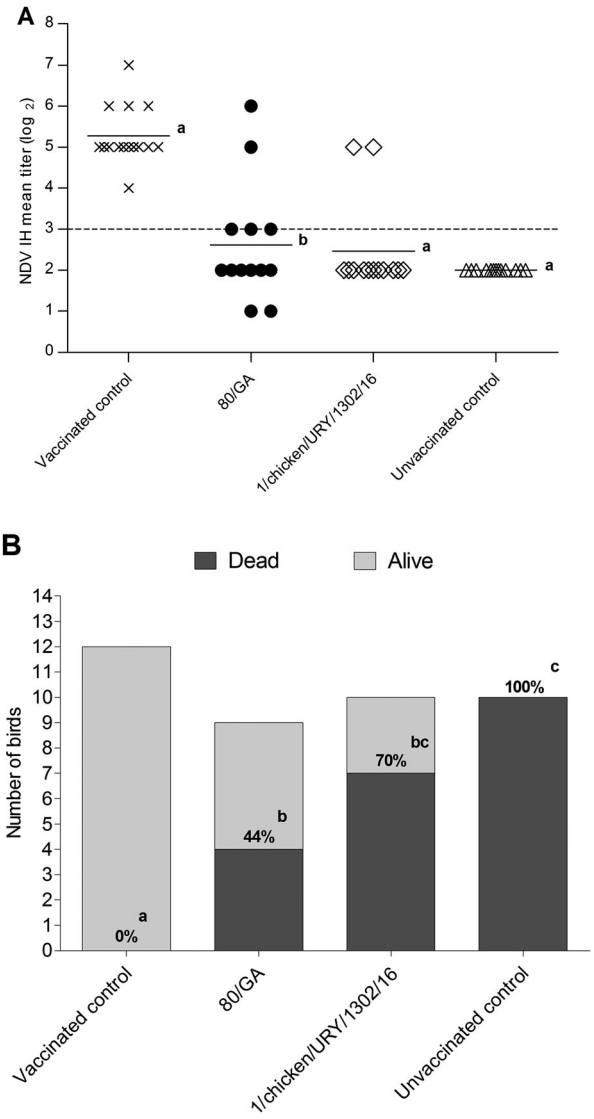


**Figure 3.** Immunosuppressive effect produced by the South American and European dIBDV strains. (A) HI mean titres observed 15 days after administration of the NDV vaccine in the vaccinated control group, European dIBDV strain (80/GA)-infected group and South American dIBDV strain (1/chicken/URY/1302/16)-infected group. Mean HI titres of unvaccinated control group were also measured. The dotted line indicates the positivity threshold. (B) Number of dead and alive chicks 15 days after infection with the NDV virulent strain Herts/33 for each group. Mortality percentages are shown. Statistically significant differences ($P < 0.05$) between selected groups are indicated with different lowercase letters.

interfered with NDV vaccinal response and demonstrate the immunosuppressive potential of this IBDV strain.

## Discussion

The emergence of classic, variant and very virulent strains are milestones in the evolutionary history of IBDV that were immediately detected by the severity of the clinical signs, mortality and histopathological lesions in the affected flocks (Cosgrove, 1962; Chettle *et al.*, 1989; Snyder, 1990). Strain emergence was strongly linked with changes in the genome that provide genetic identity to the strains and lead to their classification in genogroups (shared genetic markers) or lineages (common evolutionary origin) (Jackwood *et al.*, 2018). The characterization of lineages that diverge from traditional strains provides useful information to understand IBDV epidemiology and control. Although most novel lineages are spatially and temporally confined and should be more properly

considered as events of local differentiation, the dIBDV lineage is widespread and has persisted for several decades (Domanska *et al.*, 2004; Hernández *et al.*, 2015). Here, we achieved a complete phenotypic characterization of a dIBDV strain, recently collected from South America, to get a comprehensive view of the behaviour and impact of current dIBDV isolates for the poultry industry. Importantly, several lines of evidence support the absence of contaminating virus in the initial field sample. This sample tested negative by RT–PCR and RT-qPCR for chicken anaemia virus, infectious bronchitis virus and avian reovirus. Furthermore, seroconversion of infected chickens at 21 dpi was observed for IBDV but not for seven other common avian viral pathogens.

The dIBDV strains from South America and Europe share the same genetic (Figure 1, Supplementary material S1A and S1B) and antigenic characteristics (Table 1). The similar phylogenetic relationship inferred from the nucleotide sequences of hvVP2 and VP1 supports that dIBDV strains share similar evolutionary trends for both segments and are not reassortants. The correlation of amino acid sequences and mAb binding patterns evidences a direct link between specific residues and antigenic properties (Vakharia *et al.*, 1994; Eterradossi *et al.*, 1998; Letzel *et al.*, 2007). The lack of reactivity with mAbs 3 and 4 is related to changes within the major hydrophilic peak A, particularly changes in residues P222 and G223 (Eterradossi *et al.*, 1998). Thus, the Pro→Ser change in position 222 may explain the lack of binding of mAbs 3 and 4 in both dIBDV strains. The absence of reactivity with mAb 5 is usually observed when G254 is mutated, although reduced levels of binding occur in strains with an unaltered residue (Eterradossi *et al.*, 2004; Martin *et al.*, 2007; Soubies *et al.*, 2017). The dIBDV strains analysed here have D254, which may account for the lack of binding of mAb 5. Reduced binding of mAb 1 has not been associated with any amino acid alteration in hvVP2. It is possible that residues outside the hvVP2 region additionally affect the antigenic behaviour (Domanska *et al.*, 2004; Durairaj *et al.*, 2011). MAb 8 exhibits reduced binding affinity when some mutations occur within Loop $_{HI}$ (major hydrophilic peak B); however, this is not the case for 1/chicken/URY/1302/16 and 80/GA strains (Eterradossi *et al.*, 1998, 2004). The reduced binding affinity of the mAb 8 may also be caused by the Gly→Asp change at residue 254 due to its spatial proximity with the major hydrophilic peak B (Letzel *et al.*, 2007). All these genetic and antigenic characteristics are consistent with those reported by Domanska *et al.* (2004) with a Polish dIBDV isolate.

The comparative antigenic profiles assessed here for the dIBDV, classic virulent and very virulent strains, in addition to previously obtained results for other strains including antigenic variants (Eterradossi *et al.*, 1998; Domanska *et al.*, 2004), support that the dIBDV strains are antigenically different from traditional strains.

The genetic characteristics and phenotypic behaviour of the South American strain analysed here are similar to those observed in a strain collected in 1980 from another continent (Polish strain 80/GA); there is only one amino acid mutation between these two strains in the hvVP2 region analysed (at the position 299). Viral strains usually undergo different evolutionary processes driven by different forces, including vaccine pressures, which promotes the occurrence of neutral and selective mutations and concomitant phenotypic modifications (Boni, 2008; Chong & Ikematsu, 2017). Thus, the conservation of the dIBDV strains suggests that it has not been subjected to strong selective pressures or that it is unable to change its adaptive peak in response to evolutionary forces.

Chickens infected with the South American dIBDV strain showed subclinical infections but had a marked bursal atrophy (Figure 2(A–C)). This is in agreement with previous reports assessing the pathogenicity of Asiatic and European dIBDV strains (Minta *et al.*, 1985a; Jeon *et al.*, 2009; Yamazaki *et al.*, 2017). Thus, the absence of clinical signs seems to be an ancestral characteristic of dIBDV strains that remained unaltered over time, explaining the reduced and sporadic number of strains reported in normal clinical surveillance through the years.

Virus-induced bursal atrophy is typically associated with concomitant immunosuppression, and this was demonstrated for the dIBDV strains analysed here. The antibody levels induced by the NDV HD vaccine were reduced by around 85% and, consequently, there was a decreased protection against subsequent challenge with NDV virulent strain Herts/33 (Figure 3(A,B)). The subclinical manifestation and induced immunosuppression are characteristics shared by the dIBDV and variant IBDV strains. However, genetic analyses based on both genome segments and antigenic analyses clearly show that the dIBDV and variant strains represent different genetic lineages with different antigenic profiles (Domanska *et al.*, 2004; Hernández *et al.*, 2015; Vera *et al.*, 2015; Michel & Jackwood, 2017).

The economic impact of strains with subclinical manifestation should not be underestimated. A recent study quantified the economic losses generated by circulating strains in the period 2007–2011 in Saskatchewan province, Canada, where variant and dIBDV strains were prevalent (Zachar *et al.*, 2016). The authors concluded that the Saskatchewan poultry industry is losing around 3.9 thousand tons of meat per year, approximately 10% of its annual production. Considering that Argentina and Brazil are among the most important poultry producers, accounting for 17% of total world broiler meat production in 2017, the impact of dIBDV in these countries might be

very important and deserves a more thorough evaluation (USDA, 2018).

The protection against dIBDV strains elicited by currently used vaccines is not known. Current vaccines do not include dIBDV strains in their formulation, and may therefore have reduced protection. This is concordant with the frequent identification of dIBDV strains in Argentina and Uruguay despite continuous vaccination with classic vaccine strains, and in Canada despite the use of variant vaccine strains (Ojkic *et al.*, 2007; Hernández *et al.*, 2015; Vera *et al.*, 2015). This hypothesis is reinforced by the recent report of poor cross-neutralization between the Japanese dIBDV strain TY2 and the classic virulent strain K (Yamazaki *et al.*, 2017). *In vivo* protection assays should be done in the future to determine the level of protection of commonly used classic and variant vaccines with dIBDV strains.

In conclusion, the recent South American isolate belonging to the dIBDV genetic lineage has a particular antigenic profile, provokes a subclinical infection which results in a marked bursal atrophy, and exhibits significant immunosuppressive properties. The absence of clinical signs and mortality makes dIBDV strains phenotypically similar to variant strains. However, the genetic and antigenic divergence support that the dIBDV lineage has similar status to traditional IBDV strains and its emergence should be also considered a major evolutionary event in the IBDV history. More efforts are needed to detect and characterize dIBDV strains worldwide in order to provide new insights into their epidemiology and to determine whether specific tools are needed to control this IBDV lineage.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by the Instituto Nacional de Investigación Agropecuaria (INIA – Uruguay) under Project number 319 of the Fondo de Promoción de Tecnología Agropecuaria; the Programa de Desarrollo de las Ciencias Básicas (Uruguay); the Comisión Sectorial de Investigación Científica (Uruguay); and the Departement des Cotes d'Armor (France).

## References

Abdul, R., Murgia, M., Rodriguez-Palacios, A., Lee, C. & Saif, Y. (2013). Persistence and tissue distribution of infectious bursal disease virus in experimentally infected SPF and commercial broiler chickens. *Avian Diseases*, 57, 759–766.

Allan, W.H., Faragher, J.T. & Cullen, G.A. (1972). Immunosuppression by the infectious bursal agent in chickens immunised against Newcastle disease. *The Veterinary Record*, 90, 511–512.

Boni, M.F. (2008). Vaccination and antigenic drift in influenza. *Vaccine*, 26 (Suppl 3), C8–14.

Burkhardt, E. & Müller, H. (1987). Susceptibility of chicken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). *Archives of Virology*, 94, 297–303.

Bygrave, A.C. & Faragher, J. (1970). Mortality associated and Gumboro disease. *The Veterinary Record*, 86, 758–759.

Callison, S.A., Hilt D.A., Boynton T.O., Sample B.F., Robison R., Swayne D.E., & Jackwood M.W. (2006). Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. *Journal of Virological Methods*, 138, 60–65.

Chettle, N., Stuart, J.C. & Wyeth, P.J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. *The Veterinary Record*, 125, 271–272.

Chevalier, C., Galloux, M., Pous, J., Henry, C., Denis, J., Da Costa, B., Navaza, J., Lepault, J. & Delmas, B. (2005). Structural peptides of a nonenveloped virus are involved in assembly and membrane translocation. *Journal of Virology*, 79, 12253–12263.

Chong, Y. & Ikematsu, H. (2017). Effect of seasonal vaccination on the selection of influenza A/H3N2 epidemic variants. *Vaccine*, 35, 255–263.

Cosgrove, A.S. (1962). An apparently new disease of chickens: avian nephrosis. *Avian Diseases*, 6, 385–389.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *Journal of Virology*, 76, 2393–2402.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). Jmodeltest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T., Becht, H. & Teninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *Journal of Virology*, 32, 593–605.

Domanska, K., Mato, T., Rivallan, G., Smietanka, K., Minta, Z., de Boisseson, C., Toquin, D., Lomniczi, B., Palya, V. & Eterradossi, N. (2004). Antigenic and genetic diversity of early European isolates of infectious bursal disease virus prior to the emergence of the very virulent viruses: early European epidemiology of infectious bursal disease virus revisited? *Archives of Virology*, 149, 465–480.

Durairaj, V., Sellers, H.S., Linnemann, E.G. Icard, A.H. & Mundt, E. (2011). Investigation of the antigenic evolution of field isolates using the reverse genetics system of infectious bursal disease virus (IBDV). *Archives of Virology*, 156, 1717–1728.

Edgar, S.A. & Cho, Y. (1976). The epizootiology of infectious bursal disease and prevention of it by immunization. *Developments in Biological Standardization*, 33, 349–356.

Eterradossi, N., Arnauld, C., Toquin, D. & Rivallan, G. (1998). Critical amino acid changes in VP2 variable domain are associated with typical and atypical antigenicity in very virulent infectious bursal disease viruses. *Archives of Virology*, 143, 1627–1636.

Eterradossi, N., Gauthier, C., Reda, I., Comte, S., Rivallan, G., Toquin, D., de Boisséson, C., Lamandé, J., Jestin, V., Morin, Y., Cazaban, C. & Borne, P. (2004). Extensive antigenic changes in an atypical isolate of very virulent infectious bursal disease virus and experimental clinical control of this virus with an antigenically classical live vaccine. *Avian Pathology*, 33, 423–431.

Eterradossi, N., Picault, J.P., Drouin, P., Guittet, M., L'Hospitalier, R. & Bennejean, G. (1992). Pathogenicity and preliminary antigenic characterization of six

infectious bursal disease virus strains isolated in France from acute outbreaks. *Journal of Veterinary Medicine. Series B*, 39, 683–691.

Eterradossi, N., Toquin, D., Rivallan, G. & Guittet, M. (1997). Modified activity of a VP2-located neutralizing epitope on various vaccine, pathogenic and hypervirulent strains of infectious bursal disease virus. *Archives of Virology*, 142, 255–270.

Faragher, J.T., Allan, W.H. & Wyeth, P.J. (1974). Immunosuppressive effect of infectious bursal agent on vaccination against Newcastle disease. *The Veterinary Record*, 95, 385–388.

Giambrone, J.J. & Clay, R.P. (1986). Evaluation of the immunogenicity, stability, pathogenicity, and immunodepressive potential of four commercial live infectious bursal disease vaccines. *Poultry Science*, 65, 1287–1290.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, 59, 307–321.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704.

Hernández, M., Banda, A., Hernández, D., Panzera, F. & Pérez, R. (2006). Detection of very virulent strains of infectious bursal disease virus (vvIBDV) in commercial broilers from Uruguay. *Avian Diseases*, 50, 624–631.

Hernández, M., Tomás, G., Marandino, A., Iraola, G., Maya, L., Mattion, N., Hernández, D., Villegas, P., Banda, A., Panzera, Y. & Pérez, R. (2015). Genetic characterization of South American infectious bursal disease virus reveals the existence of a distinct worldwide-spread genetic lineage. *Avian Pathology*, 44, 212–221.

Ignjatovic, J. & Sapats, S. (2002). Confirmation of the existence of two distinct genetic groups of infectious bursal disease virus in Australia. *Australian Veterinary Journal*, 80, 689–694.

Ikuta, N., El-Attrache, J., Villegas, P., García, E.M., Lunge, V.R., Fonseca, A.S., Oliveira, C. & Marques, E.K. (2001). Molecular characterization of Brazilian infectious bursal disease viruses. *Avian Diseases*, 45, 297–306.

Ismail, N.M., Saif, Y.M. & Moorhead, P.D. (1988). Lack of pathogenicity of five serotype 2 infectious bursal disease viruses in chickens. *Avian Diseases*, 32, 757–759.

Jackwood, D.J., Schat, K.A., Michel, L.O. & de Wit, S. (2018). A proposed nomenclature for infectious bursal disease virus isolates. *Avian Pathology*, 47, 576–584.

Jackwood, D.J. & Sommer-Wagner, S. (2007). Genetic characteristics of infectious bursal disease viruses from four continents. *Virology*, 365, 369–375.

Jackwood, D.J., Sommer, S.E. & Knoblich, H. V. (2001). Amino acid comparison of infectious bursal disease viruses placed in the same or different molecular groups by RT/PCR-RFLP. *Avian Diseases*, 45, 330–339.

Jeon, W.-J., Choi, K.-S., Lee, D.-W., Lee, E.-K., Cha, S.-H., Cho, S.-H., Kwon, J.-H., Yoon, Y., Kim, S.-J., Kim, J.-H. & Kwon, H.-J. (2009). Molecular epizootiology of infectious bursal disease (IBD) in Korea. *Virus Genes*, 39, 342–351.

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874.

Kwon, H.M., Kim, D.K., Hahn, T.W., Han, J.H. & Jackwood, D.J. (2000). Sequence of precursor polyprotein gene (segment A) of infectious bursal disease viruses isolated in Korea. *Avian Diseases*, 44, 691–696.

Le Nouën, C., Rivallan, G., Toquin, D. & Eterradossi, N. (2005). Significance of the genetic relationships deduced from partial nucleotide sequencing of infectious bursal disease virus genome segments A or B. *Archives of Virology*, 150, 313–325.

Le Nouën, C., Toquin, D., Müller, H., Raue, R., Kean, K.M., Langlois, P., Cherbonnel, M. & Eterradossi, N. (2012). Different domains of the RNA polymerase of infectious bursal disease virus contribute to virulence. *PloS one*, 7, e28064.

Lejal, N., Costa, B.D., Huet, J.C., Delmas, B. & Da Costa, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *Journal of General Virology*, 81, 983–992.

Letunic, I. & Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, 44, W242–W245.

Letzel, T., Coulibaly, F., Rey, F.A., Delmas, B., Jagt, E., van Loon, A.A.M.W. & Mundt, E. (2007). Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. *Journal of Virology*, 81, 12827–12835.

Lupini, C., Giovanardi, D., Pesente, P., Bonci, M., Felice, V., Rossi, G., Morandini, E., Cecchinato, M. & Catelli, E. (2016). A molecular epidemiology study based on VP2 gene sequences reveals that a new genotype of infectious bursal disease virus is dominantly prevalent in Italy. *Avian Pathology*, 45, 458–464.

Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodríguez, J.F. & Castón, J.R. (2009). Infectious bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *Journal of Molecular Biology*, 386, 891–901.

Martin, A.M., Fallacara, F., Barbieri, I., Tosi, G., Rivallan, G., Eterradossi, N., Ceruti, R. & Cordioli, P. (2007). Genetic and antigenic characterization of infectious bursal disease viruses isolated in Italy during the period 2002-2005. *Avian Diseases*, 51, 863–872.

Mazariegos, L.A., Lukert, P.D. & Brown, J. (1990). Pathogenicity and immunosuppressive properties of infectious bursal disease "intermediate" strains. *Avian Diseases*, 34, 203–208.

McFerran, J.B., McNulty, M.S., McKillop, E.R., Connor, T.J., McCracken, R.M., Collins, D.S. & Allan, G.M. (1980). Isolation and serological studies with infectious bursal disease viruses from fowl, turkeys and ducks: demonstration of a second serotype. *Avian Pathology*, 9, 395–404.

Michel, L.O. & Jackwood, D.J. (2017). Classification of infectious bursal disease virus into genogroups. *Archives of Virology*, 162, 3661–3670.

Minta, Z., Roszkowski, J., Karczewski, W. & Kozaczynski, W. (1985a). Properties of indigenous strains of infectious bursal disease virus. II. pathogenicity. *Bulletin of the Veterinary Institute in Pulawy*, 28–29, 67–73.

Minta, Z., Roszkowski, J., Karczewski, W. & Kozaczynski, W. (1985b). Properties of indigenous strains of infectious bursal disease virus. III. immunosuppressive effect. *Bulletin of the Veterinary Institute in Pulawy*, 28–29, 73–76.

Müller, H., Scholtissek, C. & Becht, H. (1979). The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. *Journal of Virology*, 31, 584–589.

Mundt, E., Beyer, J. & Muller, H. (1995). Identification of a novel viral protein in infectious bursal disease virus-infected cells. *Journal of General Virology*, 76, 437–443.

Ojkic, D., Martin, E., Swinton, J., Binnington, B. & Brash, M. (2007). Genotyping of Canadian field strains of infectious bursal disease virus. *Avian Pathology*, 36, 427–433.

R Core Team (2014). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing: Vienna.

Reed, L.J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, 27, 493–497.

Rosales, A.G., Villegas, P., Lukert, P.D., Fletcher, O.J. & Brown, J. (1989). Immunosuppressive potential and pathogenicity of a recent isolate of infectious bursal disease virus in commercial broiler chickens. *Avian Diseases*, 33, 724–728.

Rosenberger, J.K., Cloud S.S., Gelb Jr. J., Odor E., and Dohms J.E. (1985). Sentinel bird survey of Delmarva broiler flocks. In Proceedings of the 20th National Meeting on Poultry Health and Condemnations (pp. 94–101). Ocean City, Oct. 15–16.

Rosenberger, J.K. & Gelb, J. (1978). Response to several avian respiratory viruses as affected by infectious bursal disease virus. *Avian Diseases*, 22, 95–105.

Sánchez, A.B. & Rodriguez, J.F. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology*, 262, 190–199.

Sharma, J.M., Dohms, J.E. & Metz, A.L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. *Avian Diseases*, 33, 112–124.

Shcherbakova, L.O., Lomakin, A.I., Borisov, A. V, Drygin, V. V & Gusev, A.A. (1998). Comparative analysis of the VP2 variable region of the gene from infectious bursal disease virus isolates. *Molekuliarnaia Genetika, Mikrobiologiia i Virusologiia*, 35–40.

Snedeker, C., Wills, F.K. & Moulthrop, I.M. (1967). Some studies on the infectious bursal agent. *Avian Diseases*, 11, 519–528.

Snyder, D.B. (1990). Changes in the field status of infectious bursal disease virus. *Avian Pathology*, 19, 419–423.

Snyder, D.B., Vakharia, V.N. & Savage, P.K. (1992). Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. *Archives of Virology*, 127, 89–101.

Soubies, S.M., Courtillon, C., Abed, M., Amelot, M., Keita, A., Broadbent, A., Härtle, S., Kaspers, B. & Eterradossi, N. (2018). Propagation and titration of infectious bursal disease virus, including non-cell-culture-adapted strains, using *ex vivo*-stimulated chicken bursal cells. *Avian Pathology*, 47, 179–188.

Soubies, S.M., Courtillon, C., Briand, F.-X., Queguiner-Leroux, M., Courtois, D., Amelot, M., Grousson, K., Morillon, P., Herin, J.-B. & Eterradossi, N. (2017). Identification of a European interserotypic reassortant strain of infectious bursal disease virus. *Avian Pathology*, 46, 19–27.

Spies, U., Müller, H. & Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Research*, 8, 127–140.

Stöver, B.C. & Müller, K.F. (2010). Treegraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11, 7.

Techera C., Tomás G., Panzera Y., Banda A., Perbolianachis P., Pérez R. & Marandino A. (2019). Development of real-time PCR assays for single and simultaneous detection of infectious bursal disease virus and chicken anemia virus. *Molecular and Cellular Probes*, 43, 58–60.

Tomás, G., Hernández, M., Marandino, A., Techera, C., Grecco, S., Hernández, D., Banda, A., Panzera, Y. & Pérez, R. (2017). Development of an RT-qPCR assay for the specific detection of a distinct genetic lineage of the infectious bursal disease virus. *Avian Pathology*, 46, 150–156.

USDA (2018). Livestock and poultry: world markets and trade.

Vakharia, V.N., He, J., Ahamed, B. & Snyder, D.B. (1994). Molecular basis of antigenic variation in infectious bursal disease virus. *Virus Research*, 31, 265–273.

van den Berg, T.P., Gonze, M. & Meulemans, G. (1991). Acute infectious bursal disease in poultry: isolation and characterisation of a highly virulent strain. *Avian Pathology*, 20, 133–143.

van den Berg, T.P., Gonze, M., Morales, D. & Meulemans, G. (1996). Acute infectious bursal disease in poultry: Immunological and molecular basis of antigenicity of a highly virulent strain. *Avian Pathology*, 25, 751–768.

Vera, F., Craig, M.I., Olivera, V., Rojas, F., König, G., Pereda, A. & Vagnozzi, A. (2015). Molecular characterization of infectious bursal disease virus (IBDV) isolated in Argentina indicates a regional lineage. *Archives of Virology*, 160, 1909–1921.

Xie Z., Fadl A.A., Girshick T. & Khan M.I. (1997). Amplification of avian reovirus RNA using the reverse transcriptase-polymerase chain reaction. *Avian Diseases*, 41, 654–660.

Yamazaki, K., Ohta, H., Kawai, T., Yamaguchi, T., Obi, T. & Takase, K. (2017). Characterization of variant infectious bursal disease virus from a broiler farm in Japan using immunized sentinel chickens. *Journal of Veterinary Medical Science*, 79, 175–183.

Zachar, T., Popowich, S., Goodhope, B., Knezacek, T., Ojkic, D., Willson, P., Ahmed, K.A. & Gomis, S. (2016). A 5-year study of the incidence and economic impact of variant infectious bursal disease viruses on broiler production in Saskatchewan, Canada. *Canadian Journal of Veterinary Research*, 80, 255–261.