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Research Note: Characterization and phylodynamic analysis of new infectious bursal disease virus variants circulating in Argentina

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ABSTRACT Infectious Bursal Disease is a highly contagious disease that affects young chickens and leads to significant economic losses. Its causal agent is a double-stranded RNA virus that, due to its high error rate during the replication process, gives rise to a constant generation of new virus variants. Until 2014, strains of Infectious Bursal Diseases Virus (**IBDV**) belonging to genogroup 4 predominated in Argentina, but there have been no reports since then regarding the circulating genogroups in poultry. In this study, 11 recent sequences of Argentine from the hypervariable region of VP2 protein (**hvVP2**) were analyzed to determine their genogroup, origin, evolution, and amino acid sequence. Samples from chickens showing signs of IBDV infection were collected, and the hvVP2 region was amplified using RT-PCR, followed by sequencing. The results indicated that the analyzed strains belong to genogroup 2, with an estimated evolutionary rate of 1.74×10^{-3} substitutions/site/year. It is speculated that the predominant group of sequences began to spread in Argentina around 2014 and had its origins in China. Another sample is related to strains from South Korea and is not closely linked to the main group. Furthermore, the predicted amino acid sequences show similarity to strains that can evade vaccine-induced immunity. These findings underscore the importance of active surveillance in poultry to mitigate losses caused by IBDV.

Key words: molecular epidemiology, infectious bursal disease virus, phylogenetics, phylogeography

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INTRODUCTION

Infectious Bursal Disease (**IBD**) is a highly contagious and acute disease, primarily afflicting young chickens aged 3 to 6 wk. This condition triggers humoral immunosuppression against other pathogens in the affected animals (Sharma et al., 2000). The causative agent is the infectious bursal disease virus (**IBDV**) which belongs to Birnaviridae family. Its genome is a double-stranded RNA, fragmented into 2 segments encoding five viral proteins. Among these, VP2 stands out as the key structural protein, responsible for inducing protective immunity within the host. Enclosed within amino acids 220 to 330, VP2 features a hypervariable region (**hvVP2**). This region's hydrophilic loops, identified as PBC, PDE, PFG, and PHI, have been linked to IBDV's pathogenicity and antigenicity (Reddy et al. 2022). Prior research indicates that substitutions in amino acids within hvVP2 region can lead to conformational changes in viral epitopes, enabling the virus to evade immune responses (Jackwood and Sommer-Wagner, 2011; Reddy et al., 2022).

The initial case of this disease was recorded in Gumboro, USA, in 1957, subsequently termed Infectious bursal disease due to observable morphological and histological changes within the bursa of Fabricius. The initial isolates were labeled as "classic viruses." In 1985, Delaware, USA, witnessed the emergence of strains capable of evading vaccine-derived protection from classical viruses; these were dubbed "antigenic variants" (**avIBDV**). Infectious bursal disease virus strains are categorized into seven genogroups (**G1−G7**), determined by the diversity of the hvVP2 sequence (Michel and Jackwood, 2017), with avIBDV placed under genogroup 2.

The latest genetic analysis of field isolates in Argentina indicated that the South American variant within

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genogroup 4 was responsible for most IBDV cases in the country (Michel and Jackwood, 2017). Despite this, the precise strains currently prevalent in Argentina remained undisclosed. In this context, the focal point of this study was to conduct a contemporary genetic assessment of the circulating IBDV strains in Argentina. The study was achieved using phylogeny and phylodynamics techniques to unveil the evolutionary viral history. These analyses were used before discerning inter-country relationships and transmission pathways for IBDV (Wang et al., 2022). Our study underscores the necessity of reinforcing surveillance measures to continuously monitor the circulating IBDV strains, thereby upholding the efficacy of the control strategies.

MATERIALS AND METHODS

Samples

Bursa of fabricius (**BF**) samples were gathered from chickens exhibiting IBDV infection symptoms on farms located in Entre Rios (10 samples, B15703, 15961, 16224, 16227, 16228, 16229, 16229-2, 16357, 16357-2, and 16357-3) and Buenos Aires (one sample, B16362), Argentina, in 2022.

RNA Isolation and cDNA Synthesis

Total RNA from bursal tissues was isolated using the TransZol solution (TransGen Biotech, Beijing, China) according to the manufacturer's guidelines. RNA concentration and purity were measured using a Nanodrop N100 (Thermo Scientific, Wilmington, NC) and agarose gel electrophoresis. One μg of RNA sample was reverse transcribed into cDNA using the MMLV enzyme (Promega, Madison, WI) in a 20 μL reaction mixture. The cDNA synthesis reaction was performed in a thermal cycler (Biometra, Waltham, MA) according to the manufacturer's guidelines.

IBDV Sequencing

A PCR was performed using Taq DNA Polymerase (Promega) with the following specific primers for the hypervariable region: Fw: GCCCAGAGTCTACACCAT and Rv: ATGGCTCCTGGGTCAAATCG (5′ to 3′). The amplification cycle started with a denaturation step (5 min, 95°C), followed by 35 cycles of denaturation (30 s at 95°C), primer annealing (1 min at 55°C), and extension (2 min at 72°C), and a final extension of 10 min at 72°C. The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide. The product obtained (of 579 base pairs long) was sequenced at the DNA sequencing center of the HIPRA laboratories, Province of Girona, Spain. All IBDV sequences generated here were deposited under the GenBank accession numbers OR795747 to OR795757.

Phylogeographic Reconstruction and Evolutionary Analyses

To determine the genogroup of the sequences obtained in our study, we compared them with sequences encoding the hvVP2 from Michel and Jackwood (2017) and other reference sequences. All sequences were aligned using the R package DECIPHER v2.0. The phylogenetic trees were performed using the Maximum Likelihood methodology with the IQ-Tree software version 1.4.2 (http://iqtree.cibiv.univie.ac.at). The surrogate model was estimated by means of the ModelFinder module using the same program. The tree was constructed according to the Bayesian Information Criterion with 10,000 Ultra-Fast Bootstrap replicates for test support and, finally, the results were visualized with the FigTree v.1.4.0 tool.

To determine samples evolutionary history and phylogeography, we conducted our analysis with 1200 sequences belonging to genogroup 2 of the GenBank. All sequences were aligned using the R package DECIPHER v2.0. Sequences with incomplete epidemiological information or length and indeterminate nucleotides were discarded. The presence of recombination was evaluated through the RDP4 program http://web.cbio.uct.ac.za/~darren/rdp.html using RDP, GENECONV, BOOTSCAN, MaxChi, Chimaera, SiScan and 3seq algorithms. The final alignment was composed of 436 sequences.

Subsequently, the temporal signal (time/nucleotide difference correlation) was evaluated using the TempEst software (available at https://beast.community/tempest) (Rambaut et al., 2016) and the Bayesian evolutionary analysis by sampling trees software (**BEAST** V.10.4, available at https://beast.community/) (Suchard et al., 2018) was used to estimate the Maximum Clade Credibility **(MCC)** tree, the most recent common ancestor, the mean evolutionary rate, and to reconstruct the phylogeography. The SkyRide coalescent model was employed in this study, with an uncorrelated lognormal relaxed clock model. The year and country of origin for each sequence were included in the analysis.

The Bayesian evolutionary analysis was performed twice to verify that there were no significant differences between runs and that it can be analyzed along, with 300 million Markov chain Monte Carlo iterations, using the HKY evolution model. Convergence was analyzed using the Tracer program v.1.7 and the MCC tree was drawn using the FigTree tool. To analyze and visualize the phylogeographic reconstructions, we used the SpreaD4 program (Nahata et al., 2022).

RESULTS AND DISCUSSION

Figure 1A depicts the outcome of the phylogenetic analysis involving our 11 isolates and 91 reference sequences categorized under genogroups G1 to G7, utilizing the Maximum Likelihood methodology. It was discerned that all our Argentinean isolates are classified within G2. Intriguingly, these sequences collectively form a monophyletic node exhibiting maximum support (100/100). This outcome suggests a shared origin.

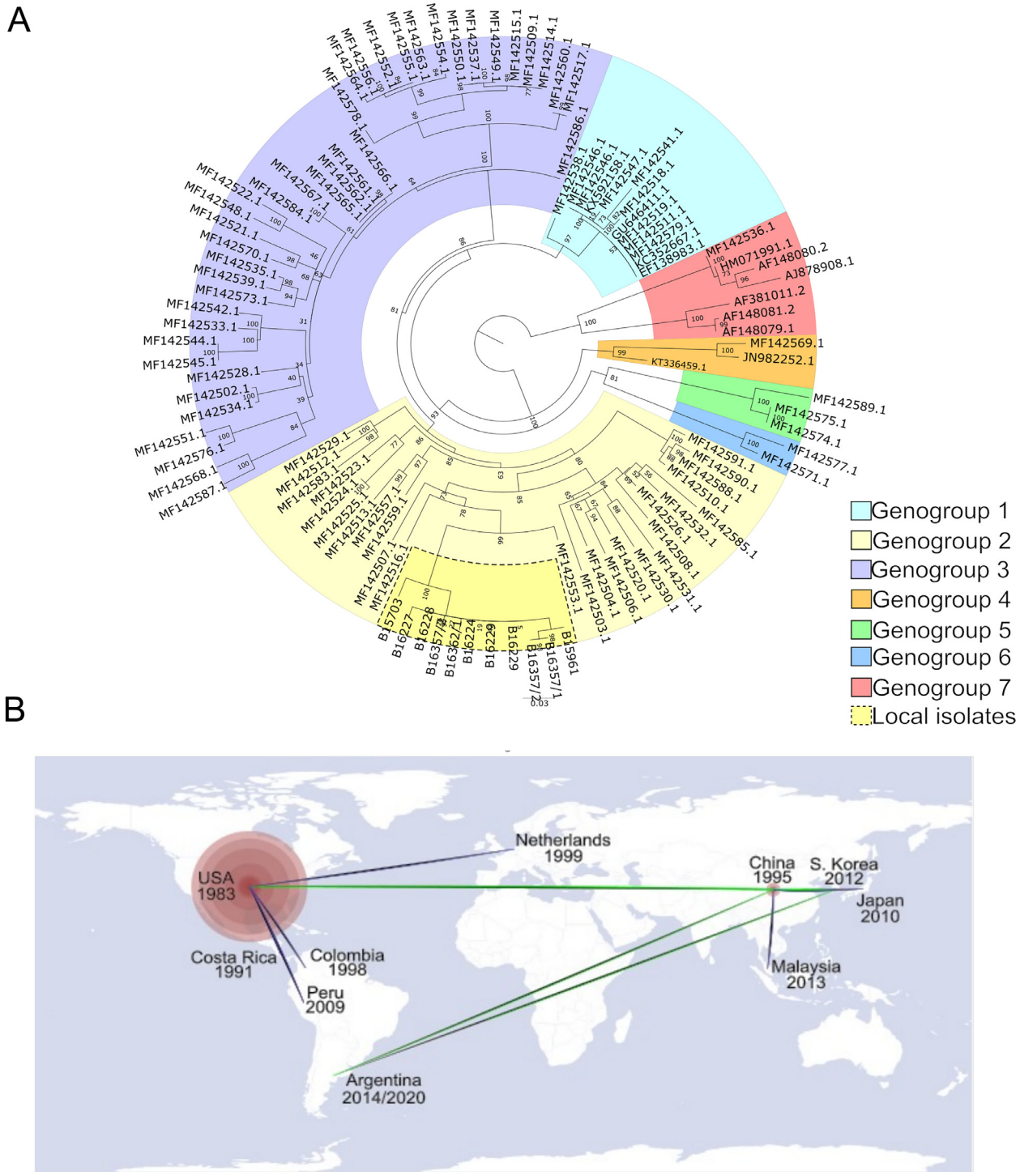

Figure 1. Phylogenetic and phylogeographic representation of migration events estimated for G2 IBDV. (A) Circular phylogenetic tree obtained by maximum likelihood. Each genogroup is indicated by a distinct color. (B) Phylogeographic reconstruction of IBDV viral dispersion. The underlined green lines indicate the possible entry routes of IBDV genogroup 2 into Argentina. The year of possible divergence of the most recent common ancestor is indicated for each country.

To investigate viral dispersion, an evolutionary and phylogeographic analysis was undertaken using a dataset containing 436 G2 sequences. A positive correlation between time and genetic divergence was observed, yielding an R-squared value of 0.59. This finding confirms the dataset suitability for subsequent Bayesian analysis. Consequently, 2 BEAST program runs were executed, and their validity was confirmed through trace plot examination. Convergence was verified by confirming that the parameter's effective sample sizes exceed 200. Our analysis unveils that the most recent common ancestor for all G2 samples originated in the United States in 1983, with a 95% highest posterior density (**HPD**) interval of 1982 to 1984. This outcome aligns with prior analyses (Wang et al., 2022), pinpointing this country as the primary source of avIBDV transmission in the 1980s. Additionally, our findings substantiate the virus's subsequent spread to China,

Table 1. Amino acid by position.

ID Sequence	215	221	222	242	249	252	254	279	286	299	309	318	321	323
Faragher 52/70	Q	Q	P	I	Q	V	G	D	T	N	K	G	A	D
Delawere	Q	Q	T	V	K	V	S	N	I	N	I	D	A	E
SHG19	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
FJ2019-01	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B15961_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16227_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16362_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16357_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16229_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B15703_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16224_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16228_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16229_2_2022	Q	K	T	V	K	I	N	N	I	S	K	D	A	E
B16357_2_2022	K	K	T	V	K	I	N	N	I	S	K	D	T	E
B16357_3_2022	K	K	T	V	K	I	N	N	I	S	K	D	T	E

Key amino acid residues in the hvVP2 region of IBDV sequences from vaccine reference strains (Faragher 52/70 and Delaware), and Argentinean (B15961-16357-3) and Chinese (SHG19 and FJ2019-01) isolates.

Japan, and South Korea, where G2 isolates have been recurrently identified.

Moreover, our modeling indicates that most Argentine viruses started spreading within the country around 2014, within a 95% HPD interval of 2013 to 2015. Notably, the sequences exhibit a close affinity with others from China. Conversely, the remaining Argentine virus follows a distinct transmission route, marked by an intermediary point between China and Argentina, with sequences reported in South Korea. This trajectory suggests an alternative potential introduction to Argentina around 2020, with a 95% HPD interval of 2019 to 2021. While part of the same clade, this last sequence does not share a direct relationship with the other 10 sequences. In any case, all eleven new Argentinean sequences belong to the previously identified lineage G2d (Wang et al. 2022). Although this study is confined to partial information due to the lack of reported sequences from several countries and their focus on a single extensively studied gene (VP2), it is plausible that the introduction of genogroup 2 to Argentina might stem from the mentioned countries or unrecorded intermediaries.

Our models have estimated an average evolution rate for all samples of 1.74×10^{-3} substitutions per site per year, with a 95% HPD spanning from 1.5×10^{-3} to 1.9×10^{-3}. The group of 10 Argentinean sequences displays a slightly higher evolutionary rate, calculated at 1.95×10^{-3} substitutions per site per year, a phenomenon not uncommon when scrutinizing samples from closely related outbreaks.

Furthermore, a comparison was conducted involving the deduced amino acid sequences of the hvVP2 region across our 11 sequences. These were compared with sequences from a classical vaccine (Faragher 52/70), a vaccine variant from genogroup 2 (Delaware), and 2 isolates from China, known for evading vaccine-generated protection (SHG19 and FJ2019-01). Key alignment positions are outlined in Table 1.

The Argentinean sequences exhibited variations, particularly in numerous residues located within the hydrophilic domains, in contrast to the vaccines. Previous work has shown that a single change, S254N aa, in the Delaware strain, was able to partially evade vaccine-generated protection (Jackwood and Sommer-Wagner, 2011). Additionally, a recent in vitro study demonstrated that the D279N substitution arises in the presence of neutralizing antibodies, substantiating antibody evasion (Asfor et al., 2022). Argentinean sequences share these 2 mutations, alongside 2 others at positions 221 and 299, with the Chinese isolates capable of evading classical vaccines-generated protection (Fan et al., 2019). When comparing our study's sequences with strains able to evade the vaccine-generated response, a 100% identity match is observed in most cases. This suggests that Argentinean strains might also elude vaccine-generated response, as variants with this amino acid sequence have previously been proven to evade vaccine-generated protection (Jackwood and Sommer-Wagner, 2011). Though incomplete, this evidence gains importance as we focus our investigation on the pivotal antigenic protein, coupled with observations of IBDV-compatible lesions in vaccinated animals. These findings raise concerns regarding the present efficacy of vaccines against circulating viruses, underscoring the urgency of active surveillance within the country's poultry farms and the imperative need to formulate vaccines that accurately represent the active circulating strains. This proactive approach aims to mitigate the economic repercussions resulting from IBD outbreaks.

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Ethical Statement: No experiment involving live animals was performed during this research.

DISCLOSURES

Authors Juan Jaton, Laura Camila Lozano, Evangelina Gómez, Guido Alberto König, and Silvina Chimeno

Zoth declare they have no financial interests. Authors Pablo Gambini and Marina Ponti have received consultant honoraria from Company HIPRA- Argentina.

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