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High antigenic diversity of serotype 1 infectious bursal disease virus revealed by antigenic cartography

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ABSTRACT

The antigenic characterization of IBDV, a virus that causes an immunosuppressive disease in young chickens, has been historically addressed using cross virus neutralization (VN) assay and antigen-capture enzyme-linked immunosorbsent (AC-ELISA). However, VN assay has been usually carried out either in specific antibody negative embryonated eggs, for non-cell culture adapted strains, which is tedious, or on chicken embryo fibroblasts (CEF), which requires virus adaptation to cell culture. AC-ELISA has provided crucial information about IBDV antigenicity, but this information is limited to the epitopes included in the tested panel with a lack of information of overall antigenic view. The present work aimed at overcoming those technical limitations and providing an extensive antigenic landscape based on original cross VN assays employing primary chicken B cells, where no previous IBDV adaptation is required. Sixteen serotype 1 IBDV viruses, comprising both reference strains and documented antigenic variants were tested against eleven chicken post-infectious sera. The VN data were analyzed by antigenic cartography, a method which enables reliable high-resolution quantitative and visual interpretation of large binding assay datasets. The resulting antigenic cartography revealed i) the existence of several antigenic clusters of IBDV, ii) high antigenic relatedness between some genetically unrelated viruses, iii) a highly variable contribution to global antigenicity of previously identified individual epitopes and iv) broad reactivity of chicken sera raised against antigenic variants. This study provides an overall view of IBDV antigenic diversity. Implementing this approach will be instrumental to follow the evolution of IBDV antigenicity and control the disease.

1. Introduction

Infectious bursal disease virus (IBDV) is the etiological agent of IBD or Gumboro disease, which is a worldwide spread immunosuppressive and sometimes lethal disease of young chickens. IBDV can infect several cell-types present in immune organs, but it shows a marked tropism for B cells from the Bursa of Fabricius (BF), a primary lymphoid organ specific to birds.

IBDV belongs to the Birnaviridae family (genus Avibirnavirus) and is a non-enveloped double-stranded RNA virus with two genomic segments. Segment A includes two partially overlapping open reading frames (ORFs). The first one codes for a non-structural protein (VP5), while the second one encodes a polyprotein precursor, which gives rise to the capsid protein (VP2), a scaffold protein (VP3) and the viral protease

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VP2 (Dey et al., 2019; Eterradossi and Saif, 2020). The hypervariable region of VP2 (VP2 HVR), which spans from residues 206 to 350 in the polyprotein, is considered as the main antigenic determinant that elicits neutralizing antibodies (Coulibaly et al., 2005; Dey et al., 2019). This variable region is located in the projection (P) domain, one out of the three capsid domains (the others designated as Base (B) and Shell (S) domains, respectively). The P domain comprises four loops (Coulibaly et al., 2005; Eterradossi et al., 1998; Eterradossi and Saif, 2020). Based on phylogenetic analyses of VP2 HVR, IBDV segment A has been classified into nine genogroups, noted from A0 to A8 (Islam et al., 2021). A putative 10th genogroup of segment A has been recently proposed after the description of recent isolates coming from Portugal (Legnardi et al., 2022). Segment B has been categorised into five genogroups, noted from B1 to B5 (Islam et al., 2021).

Studies dedicated to IBDV antigenicity have relied on two main approaches. The first one is a cross virus neutralization (VN) assay, which employs polyclonal sera raised in chickens or other species. This assay is the basis to differentiate the two described serotypes for IBDV, serotype 1 and 2 (Eterradossi and Saif, 2020; McFerran et al., 1980), where serotype 1 is the only serotype known to include both pathogenic and nonpathogenic strains. Serotype 2 strains are nonpathogenic. Cross VN assays among serotype 1 IBDV strains also allowed to identify six antigenic subtypes within serotype 1 (Jackwood and Saif, 1987).

The second approach used for the antigenic characterization of IBDV is based on panels of neutralizing monoclonal antibodies (mAbs) (Eterradossi et al., 1997; Fahey et al., 1991; Fan et al., 2020; Snyder et al., 1988a, 1992). Those mAbs were used in antigen-capture enzyme-linked immunosorbent (AC-ELISA) assays to characterize antigenic variants from the USA (Snyder et al., 1988b), Australia (Ignjatovic and Sapats, 2002) and Europe (Domanska et al., 2004; Letzel et al., 2007). These mAbs also highlighted the impact of mutations, discovered in very virulent IBDV strains, on antigenicity (Eterradossi et al., 2004, 1997; Samy et al., 2020). Additionally, several neutralizing epitopes identified thanks to mAbs were later shown to be located in VP2 HVR loops, underlining the importance of VP2 HVR for virus entry and antibody escape (Coulibaly et al., 2005).

Despite their crucial contribution to the current understanding of IBDV antigenicity, both approaches have limitations. On the one hand, VN tests have been typically performed on specific antibody-negative chicken embryonated eggs, a very time and reagent-consuming system, or in chicken embryo fibroblasts (CEF) (He et al., 2019; Jackwood and Saif, 1987; McFerran et al., 1980), a cell type that only supports IBDV replication after adaptation of the virus. This adaptation is achieved after several virus passages on CEF and requires mutations in VP2 HVR (Lim et al., 1999), some of which may affect IBDV antigenicity. Recently, chicken B cell line DT40 has been used to perform neutralization assays and rescue recombinant IBDVs (Fan et al., 2022; Reddy et al., 2022) with the absence of unwanted mutations associated to cell culture adaptation (Reddy et al., 2022). VN results may additionally depend on which species the antisera were raised (McFerran et al., 1980). Finally, antigenic differences were typically analysed using Archetti and Horsfall formula (Archetti and Horsfall, 1959; He et al., 2019; Jackwood and Saif, 1987; McFerran et al., 1980), resulting in complex datasets with sometimes difficult interpretation. On the other hand, mAbs provide a focused information about a limited number of epitopes depending on the number of mAbs used, without an overall view of their impact on antigenicity: integrating the contribution of several mAbs may lead to complex classifications (Duraiaraj et al., 2011).

To overcome those technical limitations and provide a comprehensive view of serotype 1 IBDV antigenic variation, the present study aimed at developing a new approach using several key elements. First, a two-dimensional IBDV VN assay was developed using chicken primary bursal cells (here after denoted as chicken B cells), which supports IBDV serotype 1 virus replication without prior adaptation. Second, this assay was applied to screen a panel consisting of both antigenically typical and atypical viruses belonging to 7 out of the 9 current serotype 1 genogroups for segment A and their respective post-infection chicken antisera. Third, the obtained VN data were analysed with antigenic cartography, a method previously applied for the first time to human influenza A virus (H3N2) virus (Smith et al., 2004) and since then to other viruses, such as human influenza A viruses H1N1, lyssaviruses (Horton et al., 2010), foot-and-mouth disease virus (FMDV) (Ludi et al., 2014) among others. Antigenic cartography enables reliable high-resolution quantitative and visual interpretation of large binding assay datasets (Lapedes and Farber, 2001). Results from this study provide a first comprehensive view of serotype 1 IBDV antigenic landscape.

2. Material and methods

2.1. Viruses

The sixteen IBDV serotype 1 viruses used in this work include members from 7 out of 9 genogroups for segment A (Table 1). This virus panel includes reference viral strains such as FS2/70 for genotype A1B1 (classical virulent strains) (Bygrave and Faragher, 1970), 89163 (Eterradossi et al., 1992) as reference for genotype A3B2 (very virulent (vv) strains) and variant E (varE) (Heine et al., 1991) as well as variant GLS (varGLS) (Vakharia et al., 1994) as references for genotype A2B1 (US antigen variants). 80/GA strain (genotype A4B1) was kindly provided by Dr. Jagoda Ignjatovic (CSIRO, Australia). 10144 is an Algerian reassortant strain (genotype A3B1) similar to 150124 virulent strain, with the same VP2 HVR as both 150124 and 89163 (Abed et al., 2018). Recent IBDV isolates were included in this study: D5298/1/1/20 (genotype A2B1) and D2932/3/5/3/15 (genotype A6B1), hereafter denoted as D5298 and D2932 respectively. Virus stocks were propagated in chicken B cells, as described below, at a cell concentration of 10^7/mL in 75 cm² flasks.

2.2. Isolation of primary chicken bursal cells

The sampling of Bursae of Fabricius (BF) was conducted in an animal facility approved for animal experiments (n°C-22-745-1) and approved by ANSES Ploufragan local committee for animal welfare; chickens were raised and humanely euthanized in agreement with EU directive number 2010/63/EU.

For virus stocks preparation, viral titrations and virus neutralization tests, BF were aseptically collected from four- to ten-week-old specific-pathogen-free (SPF) White Leghorns chickens (ANSES, Ploufragan, France) and were processed as previously described (Soubies et al., 2018).

2.3. Sera production

Polyclonal antisera production against varGLS, 05/5, N1/99 and 80/GA viral strains in SPF chickens was approved by ANSES ethical committee, registered at the national level under number C2EA-016/Com-Eth ANSES/ENVA/UPEC and authorized by French Ministry for higher education and research under permit number APAFIS#4945-201604131646318 v6. Antisera against varGLS, 05/5, N1/99 and 80/GA were collected from 5 to 10 five-week-old SPF White Leghorns chickens (ANSES, Ploufragan, France). Viral inocula were prepared by diluting viral stocks in a diluted made of PBS with penicillin (200 IU/mL), streptomycin (0.2 mg/mL) and fungizone (2 μg/mL) and chickens were inoculated by the intranasal route at 10^4 median tissue culture
infective doses (TCID\textsubscript{50}) in 0.1 mL per animal. After 21 days, chickens were bled and humanely euthanized.

Polyclonal antiserum production against D5298 was conducted in compliance with the provisions of Directive 2010/63/EU, Hungarian Governmental Decree No. 5/2001, (II.14.). The test facility was registered under the number 11/2015 by the Food Safety and Animal-health Directorate of the Pest County Governmental Bureau. Antiserum against D5298 was produced in 5–10 day-old broilers. Viral inoculum were prepared in PBS with a mixture of antibiotics (0.1 mg/mL gentamicin-sulfate, 0.1 mg/mL colistin-sulfate and 0.05 mg/mL norfloxacin) and animals were inoculated by the oral route at 10\textsuperscript{7} median egg infective doses (EID\textsubscript{50}) per animal. After 33 days, chickens were bled and humanely euthanatized.

Blood samples were immediately centrifuged at 1000 g for 10 min and sera were collected, pooled and transferred into clean tubes. Sera against the remaining viral strains were already available from previous pathotyping experiments performed in the authors’ laboratories (Abed et al., 2018; Felfoldi et al., 2017; Samy et al., 2020). Sera were subjected to complement inactivation by heating at 56 °C for 30 min, aliquoted in small volumes and stored at −20 °C.

### 2.4. Virus neutralization test

The dose-response relationship for a specific virus-antiserum pair was determined by a two dimension (2D) virus neutralization (VN) test based on the protocol described by Rieweyemau (Rieweyemamu et al., 1978). Serial two-fold dilutions of a specific antiserum were distributed into the columns of 96-well plates (50 µL per well). Five different virus doses (32, 100, 320, 1000 and 3200 TCID\textsubscript{50} in 50 µL per well, were added to rows (two rows for one virus dilution) and mixed with the antisera dilutions. Both antisera and viruses were diluted in Iscove's Modified Dulbecco's Medium (IMDM) with 2% SPF chicken serum

### 2.5. IBDV titration in chicken B lymphocytes

Ten-fold (for virus stock titration) or two- and four-fold (for back-titration plates) viral serial dilutions were carried out. The viral serial dilutions were distributed into 96-well U bottom plates (50 µL/well, eight replicates per viral sample). Freshly prepared chicken B cells in lymphocyte culture medium (10\textsuperscript{6} cells in 150 µL /well) were added to each well and incubated at 40 °C in a humidified 5% CO\textsubscript{2} incubator for 2 days.

After this time, bursal cells from virus titration and virus neutralization plates were washed with PBS and fixed with ethanol and acetone solution (1:1 ratio) at −20 °C for at least 30 min. After removal of the fixation solution, the plates were air-dried under a chemical hood and incubated at 40 °C for at least 30 min. After removal of the fixation solution, the plates were air-dried under a chemical hood and processed immediately or stored at −20 °C until further processing. The plates were subjected to immunochemistry (ICC) as previously described (Soubies et al., 2018). Reed and Muench formula (Reed and Muench, 1938) was used to determine viral titers expressed as TCID\textsubscript{50}/mL. Reciprocal neutralizing antibody titres were calculated and used to construct the antigenic cartography.

### 2.6. Antigenic cartography

The antigenic variation among the different IBDV genogroups was quantified and visualized using the antigenic cartography method (https://acmacs-web.antigenic-cartography.org/) (Lapedes and Farber, 2001; Smith et al., 2004). Briefly, the target distance between an antiserum A and an antigen B was determined by calculating the difference between the maximum logarithm (log\textsubscript{2}) reciprocal neutralizing titre for antiserum A against any antigen and the log\textsubscript{2} reciprocal neutralizing titre for antiserum A against antigen B. Then, the higher the reciprocal titre, the shorter the target distance, expressed as antigenic unit (AU), one AU corresponding to a 2-fold change in the titre. Using multidimensional scaling method (Kruskal and Wish, 1978), the position of each virus and antiserum in the map will be the result of minimizing the difference between the target distances and map distances. The final result is a 2D map where the distance between points represents antigenic distances as measured by VN test.

### 2.7. Antigenic relatedness by Archetti and Horsfall’s formula

The formula is given by the equation:

\[
R = \sqrt{r_A \times r_B}
\]
in which the ratio $r_2$ is determined by dividing the heterologous titer obtained with virus B by the homologous titer obtained with virus A. The ratio $r_3$ is determined by dividing the heterologous titer obtained with virus A by the homologous titer obtained with virus B. The formula yields an antibody ratio (R) that expresses the antigenic relationship between two viruses when both antigens and both sera are used in a cross VN test. A homologous R value is by definition 1. R values close to 1 indicate high antigenic similarity between two viral strains.

2.8. Antigenic study with mAbs using antigen-capture ELISA (AC-ELISA) for N1/99 and previous AC-ELISA profiles described for IBDV

AC-ELISA for N1/99 was performed as previously described (Eterradossi et al., 1997). The mAbs binding profiles for IBDV reference strains using different sets of mAbs were recovered from previous studies (see Table 2 for cognate bibliographic references) and used in this work to compare with antigenic distances obtained by antigenic cartography.

2.9. Concentration of viral stocks

Prior to RNA extraction, three millilitres for each virus stock were concentrated up to around 200 µL in Pierce™ Protein Concentrator PES, 100 K MWCO (reference 88524, Thermo Scientific) for 30 min at 3600 g and then subjected to RNA extraction.

2.10. RNA extraction, one-step RT-PCR, gel electrophoresis and purification

Viral RNAs were extracted from 140 µL of each concentrated viral stock using the QIAamp viral RNA mini kit (reference 52904, Qiagen) following the manufacturer’s instructions. Carrier RNA was replaced by linear acrylamide (reference AM9520, Thermo Fisher) at 0.025 mg/mL. RNA concentration was determined by using Qubit RNA HS assay kit (Invitrogen, Q32852) in the Qubit® 2.0 Fluorometer.

IBDV genome was reverse-transcribed into cDNA by Maxima H minus Reverse Transcriptase (reference EP0752, Thermo Fisher) according to the manufacturer’s protocol. The reaction was incubated at 50 ºC for 30 min and heated at 85 ºC for 5 min to inactivate the enzyme. cDNA was subjected to partial or full-segment PCR amplification with Phusion Hot Start II DNA polymerase (reference F549S, Thermo Fisher) following the manufacturer’s instructions. When full-segment PCR amplification failed, partial-segment PCR amplification was done. Reactions were performed as follow: 30 s at 98 ºC, 35 cycles each comprising 10 s at 98 ºC, 15 s at Tm, t (in seconds) at 72 ºC and a final step at 72 ºC for 5 min. Primer sets used during reverse-transcription and PCR, together with Tm and t for each PCR are presented in Table S1. The segments A and B from each sample were separated by 1% agarose-gel electrophoresis, eluted in ultra-pure water by Gel and PCR clean up kit (reference 740609, Macherey Nagel). DNA concentrations were measured by using Qubit dsDNA HS assay kit (Invitrogen, Q32854) in the Qubit® 2.0 Fluorometer.

2.11. Sequencing of genomic segments by next generation sequencing (NGS) and genomic data analysis

For DNA sequencing, PCR fragments were purified and quantified as described in Section 2.10. Illumina libraries were prepared with the Nextera XT kit (Illumina) according to the supplier’s recommendation. Paired-end sequencing (250 nt long) was performed on an Illumina MiSeq.

RNA sequencing was used when full and partial-segment PCR amplifications failed. NGS was performed on the RNA extract after rRNA depletion with the Low Input Ribominus Kit (Ambion), as described by the manufacturer. A RNA library was obtained using Ion total-Seq Kit v2 (Life Technologies) according to the manufacturer’s recommendations and was then sequenced with using Ion Torrent proton technology (Life Technologies).

Data were analysed using Geneious version 2020.2.4 software (Biomatters Ltd, Auckland, NZ) (Kearse et al., 2012). Sequence Reads obtained by RNAseq were directly imported into Geneious. For each sample sequenced by RNAseq, the two paired .fastq files generated on the MiSeq platform were imported into Geneious and paired. Then, all sequences were trimmed as part of the assembly process using BBDuk trimmer tool. Trimmed reads were then mapped against an annotated reference sequence for segment A or B (GenBank accession n’ HG974563.1 or HG974564.1, respectively) with the Geneious Mapper with medium sensitivity and 5 fine-tuning iteration parameters. A consensus sequence was generated after mapping with a default base threshold of the highest quality and for reads without a quality score at a particular base, a default threshold of 65% was used to call the consensus. Variants were called using the Geneious variant finder at a cut-off frequency of 10%. The data were deposited in the BioSample database at NCBI under GenBank accession numbers from ON100649 to ON100679.

2.12. Alignment and phylogenetic tree

Nucleotide and amino acid alignments from the generated consensus sequences were done using the Clustal W algorithm in MEGA Software version 7 (Kumar et al., 2016). Phylogenetic analyses were performed using published nucleotide sequences (Islam et al., 2021) that were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/). The analyses were made on nucleotide and amino acid sequences using neighbor joining method based on the Kimura 2-parameter model (when nucleotide sequences were used) or Poisson model (when amino acid sequences were used) with 1000 bootstrap replicates implemented in MEGA software version 7. Bootstrap values lower than 75% were considered non-significant. Phylogenetic relationships were based on a 517 base pairs (bp) fragment in the VP2 HVR in segment A (from nucleotide 546 to 1062 corresponding to amino acid 177 to position 360 in the polyprotein) and a 474 bp fragment in segment B (from nucleotide 385 to 859, amino acid 100 to position 257 in VP1). For segment B, the region selected overlapped with the B marker region considered to be phylogenetically representative (Alfonso-Morales et al., 2015; Islam et al., 2021).

2.13. Statistical analysis

In order to represent the VN data in the antigenic map, Racmacs 1.1.35 package was used (R software, version 4.1.1.). To determine the optimal number of dimensions for visualizing the VN data, a prediction test was undertaken. For this purpose, antigenic maps were made with 10% of the titers omitted at random. Those omitted values were then predicted using maps with dimensions varying from two to five.

Clusters in the antigenic map were identified by a K-means clustering algorithm. In order to compare the variance among virus-antiserum distances obtained from the antigenic cartography for each serum, a Barlett’s test was performed, followed by post-hoc pairwise comparison using the “RVAideMemoire” library version 0.9-81-2.

3. Results

3.1. Phylogenetic analysis based on nucleotide and amino acid sequences

To confirm the identity of each virus used in the present work and to characterize genetically two new isolates, D5298 and D2932, the sixteen viral stocks listed in Table 1 were completely or partially sequenced by NGS and the data were subsequently used in phylogenetic and variant analysis (see Section 3.2). The phylogenetic analyses for segment A and B were performed with 96 and 77 sequences respectively, including in
Table 2
Antigenic study of N1/99 with mAbs and previous antigenic characterization by Antigen-Capture ELISA (AC-ELISA) of IBDV serotype 1 viruses used in the present work.

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The reactivity with each mAb is showed in black boxes. Reduced binding or lack of neutralization are evidenced as blank shading in the table.

*Similar to 150144

(nt: no tested)
both cases the sixteen sequences from the viruses listed in Table 1 (Fig. S1A-B). For F52/70, segment B sequence was downloaded from GenBank (accession no. HG974566.1). To help the reader, the phylogenetic trees were simplified and showed in Fig. 1A-B. For both IBDV segments, sequence data from viruses previously described grouped consistently as indicated in previous works (Fig. 1A-B) (Islam et al., 2021; Michel and Jackwood, 2017). For segment A, D5298 and D2932 clustered with genogroup A2 and A6 respectively. D5298 presented three out of four typical amino acids conserved in US variants in VP2 HVR (V242, K249 and N279) (Domanska et al., 2004), with an Alanine (instead of Q/T) at position 222 (Fig. 2). D2932 presented in VP2 HVR similar residues to those present in other strains in A6 genogroup (Q222, E253, S253 and V321) (Felföldi et al., 2017; Lupini et al., 2016) (Fig. 2). For segment B, both isolates clustered with genogroup B1 (Fig. 1B). When a phylogenetic tree was made for segment A and B based on amino acid sequence of VP2 HVR sequences (Fig. 1C-D), the sixteen viruses grouped again in their respective genogroups.

3.2. Nucleotide variant analysis by NGS in the VP2 hypervariable region

To address the effect of the possible presence of nucleotide variants on cross-neutralization, the composition of viral population in the VP2 HVR was determined. Table 3 shows the variant frequencies found in each virus used in the present study. Viral stocks generally appeared genetically homogenous with few mutations presenting a frequency above an empirical 10% threshold. Two non-synonymous changes were found in 80 G/A VP2 at amino acid positions 222 and 279 with variant frequencies of 11 and 14.8%, respectively.

Analysis of F52/70 viral stock initially chosen for the study revealed the presence, with a frequency of 39.2% of a P222T subpopulation.

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**Fig. 1. Phylogenetic trees of segments A and B.** (A, B) Phylogenetic trees of analyzed viruses based on nucleotide sequences for segment A (A) and B (B). Genogroups are indicated in dashed squares. See Fig. S1 for complete trees used to determine studied viruses genotype. (C, D) Phylogenetic trees based on VP2 hypervariable region (HVR) amino acid sequences (positions 177–360) for segment A (C) and based on a 474 bp sequence (amino acid positions 100–257) for segment B, corresponding to the VP1 N-terminal domain and the finger subdomain of the central polymerase (D). The trees based on amino acid sequences were generated using Neighbor Joining method based on Jones-Taylor-Thornton (JTT) model with 1000 bootstrap replicates implemented in MEGA software version 7. Bootstrap values >75% are indicated.
Another stock, hereafter named antibody neutralization-escape mutants. Fig. 2. L.L. Cubas-Gaona et al. F52/70 P222T be without detectable subpopulation. The first stock, hereafter named corresponding to 100 TCID excluded from the analysis. Importantly, the neutralizing antibody titre (Rweyemamu et al., 1978); those aberrant values were therefore dose-response curves for F52/70 as an example), except for some dose-response curves were usually rectilinear (Fig. S2 shows only the listed in Table 1 using a two-dimensional neutralization test. The were obtained for each of the five doses of virus from the sixteen viruses chicken B cells, the respective neutralizing antibody titres of eleven sera neutralization tests \( \frac{\text{R}}{\text{w}} \text{e}\text{y}\text{e}\text{y}\text{e}\text{m}\text{a}\text{m}\text{u}\text{u}\text{m}\text{u}\text{a}\text{u}\text{,} \text{1978} \). Then, in order to characterize this variant on antigenicity. Table S2 summarizes the coefficient of correlation, \( \alpha \text{-}00154, \alpha \text{-}D2932 and \alpha \text{-}80/GA, \alpha \text{-}D2932 and N1/99 formed a second cluster (in gray). VarE, AVS-MB, 91168, D3976 and 00154 belonged to a first cluster (in green). 80/GA, D3976 and 00154 were the most always appeared on the linear part of the curve, demonstrating the linear dependence between neutralizing anti
titers and virus dose. Thus, neutralizing antibody titers were calculated for 100 TCID\(_{50}\) always appeared on the linear part of the curve, demonstrating the linear dependence between neutralizing antibody titer and virus dose. Rvalues from Archetti and Horsfall formula have been historically used to quantify antigenic relatedness between two viral strains. Then, \( R \) values (Table S3) were calculated from VN test data (Table 4) for 11 out of 16 viruses as this formula can only be applied when homologous antiserum is present, and compared with the antigenic distances. Both metrics showed a negative correlation (Spearman’s correlation coefficient of \( -0.73 \)) (Fig. 3B), where antigenic distances from 2.5 units upwards corresponded to \( R \) values below 0.5.

3.3. Dose-response relationships for homologous and heterologous viral neutralization tests

The dose-response relationships of homologous and heterologous virus-antiserum pairs have been defined as the criterion to determine significant differences between viral strains (Booth et al., 1978; Rweyemamu et al., 1978). Then, in order to characterize this dose-response relationship for a specific virus-antiserum pair on primary chicken B cells, the respective neutralizing antibody titres of eleven sera were obtained for each of the five doses of virus from the sixteen viruses listed in Table 1 using a two-dimensional neutralization test. The dose-response curves were usually rectilinear (Fig. S2 shows only the dose-response curves for F52/70 as an example), except for some virus-antiserum pairs at high virus inputs, as previously described (Rweyemamu et al., 1978); those aberrant values were therefore excluded from the analysis. Importantly, the neutralizing antibody titer corresponding to 100 TCID\(_{50}\) always appeared on the linear part of the curve, demonstrating the linear dependence between neutralizing antibody titer and virus dose. Both viruses and antiserum can be displayed in an antigenic cartography map, as presented for the current dataset in Fig. S3. To make map interpretation easier and avoid overplotting, Fig. 3A only shows the sixteen viruses used in the present work.

3.4. Antigenic relatedness by antigenic cartography

Antigenic cartography provides a quantitative and visual representation of binding assay data. Therefore, antigenic cartography was used to further characterize the antigenic relatedness among all viruses used in this work using the VN values from Table 4. A prediction test was carried out to evaluate the reliability of the cartography and the optimal number of dimensions to represent the dataset. Similar mean prediction errors were found for each dimension \( 1.378 \pm 0.119 \) antigenic units (AU) for the second dimension, \( 1.322 \pm 0.159 \) AU for the third dimension, \( 1.342 \pm 0.155 \) for the fourth dimension and \( 1.352 \pm 0.154 \) AU for the fifth dimension], suggesting no discernible mean advantage in precision using higher dimensions. The final antigenic map was therefore represented in 2D. Both viruses and antiserum can be displayed in an antigenic cartography map, as presented for the current dataset in Fig. S3. To make map interpretation easier and avoid overplotting, Fig. 3A only shows the sixteen viruses used in the present work.

3.4.1. Correlational study between antigenic cartography and Archetti and Horsfall approaches

\( R \) values from Archetti and Horsfall formula have been historically used to quantify antigenic relatedness between two viral strains. Then, \( R \) values (Table S3) were calculated from VN test data (Table 4) for 11 out of 16 viruses as this formula can only be applied when homologous antiserum is present, and compared with the antigenic distances. Both metrics showed a negative correlation (Spearman’s correlation coefficient of \( -0.73 \)) (Fig. 3B), where antigenic distances from 2.5 units upwards corresponded to \( R \) values below 0.5.

3.4.2. Antigenic distances in the 2D IBDV map

Antigenic distances from 2D map among IBDV strains were calculated and represented in Table 5. The antigenic map revealed that F52/70 and F52/70 P222T within A1 genogroup are in close vicinity (0.57 AU), indicating that the 222T subpopulation does not seem to have a strong impact on F52/70 antigenicity. Both viruses were antigenically related to viruses in A3 genogroup, with a distance from viruses belonging to the A3 genogroup ranging from 0.47 to 1.75 antigenic units (AU). Within the A3 genogroup, 89163, 150144 and 91168 appeared antigenically closely related, showing negligible antigenic distance between 89163 and 150144 (0.25 AU). D3976 and 00154 were the most distant within this genogroup with 1.24 and 1.4 AU from 89163, respectively. Viruses belonging to the A2 genogroup grouped together and were antigenically distant from the others genogroups, except for VarGLS, which was antigenically closer to 05/5 (0.23 AU) than to its own genogroup. Four antigenic clusters were distinguished using a K-means clustering algorithm: F52/70, F52/70 P222T, 89163, 150144, 91168, D3976 and 00154 belonged to a first cluster (in green). 80/GA, D2932 and N1/99 formed a second cluster (in gray). VarE, AVS-MB, AL2-like and D5298 belonged to a third cluster (in orange) while...
VarGLS and 05/5 were grouped into a fourth cluster (in pink) (Fig. 3A). In addition, antigenic distances between sera and antigens were calculated. Sera against variant viruses (in particular anti-varGLS, anti-varE, anti-05/5 and anti-D5298) showed significantly lower variance in their antigenic distances to viruses as opposed to anti-classical virus sera (such as anti-F52/70 or anti-N1/99) (Fig. 3C and Table S4). This is in agreement with raw neutralizing titers that appear to vary less for sera raised against variant viruses (Table 4). These data collectively indicate a broader cross-reactivity of anti-variant sera.

3.5. Antigenic study with mAbs for N1/99 and previous mAb binding pattern for IBDV: comparison with antigenic cartography

Several mAb sets have been produced to characterize the antigenic diversity of IBDV. In order to evaluate the contribution of antigenic cartography in the complete antigenic characterization of IBDV, the authors have considered instrumental to compare published mAb binding profiles with IBDV antigenic relatedness obtained from antigenic cartography. Table 2 shows a summary of these mAbs binding patterns to help the reader.

3.5.1. Antigenic comparison with mAbs 1, 3, 4, 5, 6, 7, 8 and 9

Even though F52/70 and 89163 binding patterns using mAbs previously produced by Eterradossi et al. (Eterradossi et al., 1997) are different (lack of reactivity with mAbs 3 and 4 for 89163), a small antigenic distance was observed between F52/70 and 89163 (0.57 AU, \( R = 0.89 \)), which is consistent with previous results obtained with egg-based VN (Eterradossi et al., 1992). In addition, the extra lack of reactivity with mAb 8 of the atypical vvIBDV 91168 (Eterradossi et al., 1998) was not translated in a major change for this atypical vvIBDV in the antigenic map compared with F52/70 and 89163. The identical mAbs binding pattern of 89163 and 150144 was in agreement with i) the almost identical position in the antigenic map (distance of 0.1 AU) and ii) the identical HVR sequence of these viruses.

On other hand, the markedly reduced antibody binding profile of 00154 (lack of reactivity with mAbs 3, 4, 5, 6, 8, and 9) (Samy et al., 2020) was translated in 1.75 AU from F52/70 (\( R = 0.48 \)). Likewise, the lack of reactivity with mAb 1 of 80/GA (also lack of reactivity with mAbs 3, 4, 5 and 8 (Domanska et al., 2004; Tomás et al., 2019)) and N1/99 was translated in even greater antigenic distances from F52/70 (3.12 and 1.97 AU, respectively).

3.5.2. Antigenic comparison with mAbs 57, R63, 67, B69 and 10

Antigenic properties of reference US variants IBDV (varE and varGLS) have been also characterized using mAbs 57, R63, 67, B69 and 10 (Letzel et al., 2007; Snyder, 1990; Snyder et al., 1988a). Both strains exhibited very different antigenic profiles, which is consistent with the 4.8 AU (\( R = 0.11 \)) in the antigenic map between varE and varGLS, indicating they are very distant antigenically speaking. The lack of reactivity (when test was possible) with mAbs developed by Eterradossi et al. is in agreement with the antigenic map, as varF and varGLS are distant from viruses in genogroups A1, A3, A4 and A7, which reacted at least with two mAbs.

3.5.3. Antigenic comparison with mAbs 17-82, 39A, 44-18 and 9-6

Antigenic properties of reference US variants IBDV (varE and varGLS) have been also characterized using mAbs 57, R63, 67, B69 and 10 (Letzel et al., 2007; Snyder, 1990; Snyder et al., 1988a). Both strains exhibited very different antigenic profiles, which is consistent with the 4.8 AU (\( R = 0.11 \)) in the antigenic map between varF and varGLS, indicating they are very distant antigenically speaking. The lack of reactivity (when test was possible) with mAbs developed by Eterradossi et al. is in agreement with the antigenic map, as varF and varGLS are distant from viruses in genogroups A1, A3, A4 and A7, which reacted at least with two mAbs.

3.5.4. Antigenic comparison with mAbs 17-82, 39A, 44-18 and 9-6

It is well known that Australian classical and variant IBDV strains are distinct groups compared with classical, very virulent and US variant IBDV based on genetic sequence (Ignjatovic and Sapats, 2002; Sapats and Ignjatovic, 2000). The panel of mAbs developed by Faehey et al. (17–82, 39A, 44-18 and 9-6) has allowed to differentiate between both Australian variant and classical ones (Faehey et al., 1991). The very different binding patterns observed between N1/99 (reactivity with all mAbs) and 05/5 (lack of reactivity against 17-82, 39A and 44-18) (Ignjatovic and Sapats, 2002) mirrored their separation in the antigenic map, with a distance of 3.28 AU (\( R = 0.08 \)).
Table 4
Neutralizing antibody titres upon cross-VN test between the sixteen IBDV and eleven IBDV infected chicken sera used in the present work.

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<th>α-D5298</th>
<th>α-89163</th>
<th>α-00154</th>
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Titres are expressed as the calculated reciprocal of serum dilution which neutralized 100 TCID_{50} of virus. Homologous titres are in bold.

Fig. 3. Antigenic relatedness by Antigenic Cartography. (A) Two-dimensional (2D) antigenic map of the sixteen viruses used in the present work against eleven sera from IBDV infected chicken. The positions of these viruses were based on the neutralizing titre at a virus dose of 100 TCID_{50}. Colors represent the antigenic clusters identified by a K-means clustering algorithm. The vertical and horizontal axes both represent antigenic distances. The space between grid lines is 1 unit of antigenic distance, corresponding to a two-fold dilution of serum in the VN test. B. Correlation between antigenic distances and R values (Archetti and Horsfall). The antigenic distances between viruses were derived from the 2D antigenic map and the R values from the neutralizing antibody titres. Spearman’s correlation coefficient was -0.73 (P < 6.2 × 10^{-11}). C. Analysis of variance for serum-virus distances. A Bartlett’s test was used for variance comparison of serum-virus distances for each serum. Different letters indicate significant difference (p < 0.05) between the variances.
4. Discussion

The present study provides, for the first time, an overall view of IBDV serotype 1 viruses antigenic variation in an antigenic cartography using a panel of sixteen serotype 1 IBDV's from four continents, eleven chicken post-infectious antisera and a 2D VN test performed on primary chicken B cells. The panel of viruses used in the present work included viruses considered like historic reference virus strains, viruses with antigenic variations previously characterised using mAbs, as well as recent isolates associated with problems in the field (DS298 and D2932).

Unlike chicken fibroblasts, primary chicken B cells appear as a versatile cellular system to carry out cross VN test with serotype 1 IBDV viruses regardless their capacity to replicate in classical cell culture systems. Additionally, primary chicken B cells system appears to be a sensitive system for VN tests since up to 1000-fold differences in VN titres were detected between different viruses for a given antiserum. Similar differences were previously seen for IBDV typing using CEF cultures (He et al., 2019; Sapats and Ignjatovic, 2000) and, recently, using chicken B cell line DT40 (Fan et al., 2022; Reddy et al., 2022).

In contrast with Archetti and Horsfall values, antigenic cartography enabled determining antigenic relatedness among a panel of viruses using multiple sera simultaneously, even in the absence of homologous antiserum, resulting in a more flexible implementation. Antigenic cartography showed high antigenic relatedness between N1/99, D2932 and 80/GA despite these viruses belonging to different genogroups based on VP2 HVR phylogenetic analyses. This highlights that genetic data, although instrumental in IBDV molecular epidemiology, may be misleading when used to infer antigenic relatedness, similarly to what is observed with other avian viruses such as infectious bronchitis virus (de Wit et al., 2011).

Conventionally, IBDV serotype 1 viruses have been antigenically classified as classical or antigenic variants (Eterradossi and Saif, 2020). However, the antigenic map indicates that this binary classification may be restrictive and that more than two antigenic groups may be distinguished. This result is consistent with earlier studies which already noted the antigenic distance among them (Jackwood and Saif, 1987). The close antigenic relatedness between classical (A1) and very virulent (A3) viruses has been previously described (Brown et al., 1994; Eterradossi and Saif, 2020). This is in agreement with the antigenic map, where these viruses were displayed in close vicinity. mAbs binding patterns have been used to characterize IBDV antigenic properties. The loss of reactivity with mAbs 3, 4 and 8 has been associated with changes in amino acid positions 222, 223 and 324 (Eterradossi et al., 1998), respectively. These epitopes seem to have a modest contribution to IBDV overall antigenicity, as illustrated by i) the small distance of 0.57 AU observed between F52/70 and the mixed population F52/70 P222T stock (mutation frequency of 39.2%), ii) the 0.57 AU between F52/70 and strains 89163 and 150124 (associated to loss of mAb 3 reactivity) or iii) the 0.47 AU between F52/70 and strain 91168 (loss of mAb 8 recognition). These results are consistent with previous results from the authors’ laboratories showing that a laboratory selected serotype 1 IBDV virus, escaping neutralization by mAbs 3, 4, 6, 7 and 8, was still efficiently neutralized (14 log2) by an antiF52/70 antiserum (unpublished).

In contrast, the higher distance (1.97AU) between F52/70 and N1/99 was associated to the sole lack of reactivity with mAb1. This indicates a major contribution of the unknown epitope recognized by mAb 1 to the overall antigenic profile, as compared with the other mAbs described by (Eterradossi et al., 1997).

Mutations in the Loop P_{HI}, which includes 321 amino acid position, have been reported to be critical in neutralization escape mutants (Coulibaly et al., 2005; Samy et al., 2020). The important lack of reactivity of 00154 with mAbs developed by Eterradossi et al. has been related with three unusual mutations compared with 89163 (Y220F, G254S and A321T) (Samy et al., 2020). Similarly, S254 and V321 residues from isolate D2932 could explain its reduced antigenic relatedness with viruses from genogroups A1 and A3 in the antigenic map, since position 254 has also been previously described as important for antigenicity. Although multiple epitopes contribute to the overall antigenicity, mutation at position 321 in 00154 and D2932 could play an important role in these antigenic variations. On the other hand, the glutamic acid (E) at position 321 in variant GLS could partially explain its position in the antigenic map. The presence of glutamate (E) has been described as necessary for the reactivity against mAb 67 but not with mAb 57 (Letzel et al., 2007). However, AL2-like having a E at position 321, would indicate that other additional residues could be also responsible for varGLS position such as D213, which has been involved in the escape from neutralizing monoclonal antibodies (Heine et al., 1991).

No antigenicity studies have been previously carried out with the subclinical reassortant D3976, that together with 00154 appeared, within the A1 and A3 genogroups, as the most distant from F52/70 in the map. Four mutations in VP2 HVR, have been described in D3976 compared with 89163: Q219L, G254D, D279N and N280T (Mató et al., 2020), among which the G254D change is also present in 80/GA (Domanska et al., 2004; Jackwood and Sommer-Wagner, 2011). This G254D in 80/GA as well as P289 and I290 residues, which have been previously described to be involved in antigenicity, could also explain its particular mAb binding profile and its marked antigenic distance from F52/70 and 89153.

When looking at the two Australian isolates used in this work, only the classical N1/99, aligned and mapped with classical IBDV viruses...
while the other, the Australian variant 05/5, diverged from classical and vv IBDV strains (as reported by Brown et al. (Brown et al., 1994) for an earlier isolate, 002-73) and mapped as far away as the US variants. Although American and Australian antigenic variants are very different genetically speaking, the most antigenically related strain with 05/5 was varGLS (0.23 AU) and both strains belong to the same antigenic cluster. Although the reason of this high antigenic relatedness despite genetic differences is not clear, the presence of different residues at position 321 (T321 in 05/5, E321 in varGLS) compared with F52/70 could provide a partial explanation. It could be further speculated that similar evolutionary pressures, namely the use of antigenically classical vaccines, resulted in the independent appearance of these viruses.

In general, good correlation existed for the antigenic relatedness between strains, as deduced by either panel of mAbs or by antigenic cartography. However, the antigenic map showed in the present work provides an overall and more quantitative view of IBDV antigenic landscape as cross protection events can be studied. Also, the capability to define antigenic cluster allows to identify differences at amino acid level between antigenic groups. It has been key in the monitoring of antigenic variations in circulating viruses and updates of vaccines in viruses such as FMDV and influenza A viruses (Lerussu et al., 2013; Ludi et al., 2014; Smith et al., 2004). Another strength of the antigenic map is its easier interpretation of antigenic data since visualization is possible.

An additional interest of antigenic cartography is the mapping of sera to evaluate their cross-reactivity. The higher a distance between a serum and a virus, the lower its neutralizing titer towards this virus. Consequently, while some sera (for example anti-F52/70) appeared highly specific but with a narrow reactivity spectrum, others, especially antivariant sera, displayed broad cross-reactivity as indicated by i) their central position in the antigenic map and ii) the lower variance of their distance to viruses. This fact is in agreement with the literature where sera obtained from chickens infected by varE could protect against viruses belonging to classical, very virulent and variant strains (Eterradosi and Saïf, 2020; Mundt et al., 2003). IBDV antigenic cartography could thus help identify field strains eliciting such broadly-reactive sera as potential vaccine candidates.

The map obtained in this work provides a view of IBDV natural antigenic landscape as chicken post-infectious sera have been used. Since IBD is a disease of young chickens, maternally-derived antibodies represent the unique line of defense against field viruses during the first days post-hatching. Then, post-vaccinal sera would be of interest to test in order to determine the level of protection of a given vaccination program.

In the future, any new virus may be screened against the current sera panel to enrich the antigenic cartography. If only sequence information is available for the virus, VP2 HVR sequence information could be sufficient to rescue a chimeric, recombinant virus expressing the VP2 HVR to be tested, as performed by others (Durairaj et al., 2011; Reddy et al., 2022).

Similarly, reverse genetics will be instrumental to dissect the contribution to antigenicity of individual mutations, especially those located within VP2 HVR.

The VN test is a powerful tool to predict antigenic relatedness and allows for the typing of IBDV isolates and can be used to predict differences in immunogenicity. However, this approach should be confirmed by in vivo cross-protection assays.

5. Conclusion

The antigenic characterization of IBDV through antigenic cartography using VN data employing primary chicken B cells has allowed not only to have a more extensive antigenic landscape for serotype 1 IBDV viruses, but also overcome some technical limitations as no previous IBDV adaptation is required. This work is a proof-of-concept of the use of antigenic cartography and this approach should represent an innovative tool in the control of IBD.

Funding

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Fig. S1. Phylogenetic trees of segments A and B. For segment A, phylogenetic tree was based on 96 selected representative strains of IBDV based on VP2 hypervariable region (HVR) sequences (nt 546-1062). B. For segment B, phylogenetic tree was based on 77 selected representative strains of IBDV based on the 474-bp sequence corresponding to the VP1 N-terminal domain and the finger subdomain of the central polymerase (nt 385-859). The trees were generated using Neighbor Joining method based on the Kimura 2-parameter model with 1000 bootstrap replicates implemented in MEGA software version 7. Bootstrap values:>75% were indicated. The trees were drawn to scale. The IBDV strains used in the present work are indicated by filled circles.

Fig. S2. Representative dose-response relationship for homologous and heterologous viral neutralization tests for anti-F52/70. Serum and virus titres are represented in log2 and log10, respectively.

Fig. S3. Two-dimensional antigenic map of IBDV serotype 1 viruses showing both antigen and sera positions. Two-dimensional (2D) antigenic map of the sixteen viruses (round shape) used in the present work against eleven sera (square shape) from IBDV infected chicken. The positions of these viruses were based on the neutralizing titre at a virus dose of 100 TCID50. For viruses, colors represent the antigenic clusters identified by a K-means clustering algorithm. The vertical and horizontal axes both represent antigenic distances. The space between grid lines is 1 unit of antigenic distance, corresponding to a two-fold dilution of serum in the VN test.

CRediT authorship contribution statement


Declaration of Competing Interests

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198999.
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