# Molecular epidemiologic survey of infectious bursal disease viruses in broiler farms raised under different vaccination programs

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**Primary Audience**: Researchers, Extension Services, Veterinarians

## SUMMARY

Over a span of nearly 4 yr, 246 bursal tissue samples were collected from Brazilian commercial broiler flocks (*Gallus gallus*) throughout the country and imprinted to sample collection cards (Flinders Technology Associates (FTA) cards). A total of 75 infectious bursal disease virus (IBDV) strains was successfully detected from the FTA card imprints and were submitted for further identification and molecular characterization. Nucleotide and predicted amino acid sequences of the IBDV surface protein VP2 were used to identify strains of the virus and place them into phylogenetic groups. The amino acids across the hypervariable region of VP2 in this study varied, but around half of all positive samples were classified as vaccine virus. The IBD viruses fell into 3 categories: variant IBDV, classic IBDV (vaccine), and very virulent (vv) IBDV. The samples were collected according to the 3 different vaccination strategies used in broilers: vectored vaccine, antigen-antibody complex vaccine, and conventional live vaccine. The genetic profile and frequency of the strains recovered from the flocks were highly dependent on the vaccination program. This information helps us gain a better understanding of the current landscape of IBD in Brazil and provides additional scientific data to support selection of the most effective vaccination strategies, products, and practices to prevent disease.

**Key words:** gumboro, vaccination programs, poultry, epidemiology

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## DESCRIPTION OF PROBLEM

Vaccination with live attenuated virus is used worldwide to control infectious bursal disease (**IBD**) in commercial poultry flocks. However, effective control using conventional live vaccines requires proper timing of field vaccination based on serological monitoring of maternal antibody levels. While on the one hand, the live vaccine is susceptible to neutralization by high maternal antibodies [1], there is also the potential for immunosuppression if flocks are vaccinated when they are young or if their antibody levels are too low [2–3]. Different vaccine strains vary with regard to these issues [4], which is why producers have more frequently turned to immune complex and recombinant vaccines that can be administered safely and

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effectively from the hatchery. Today, these 2 types of IBD vaccines are used to control IBD on most Brazilian farms. Although IBD occurs in Brazil, both technologies have generally been effective in controlling clinical disease. Live vaccines of the immune complex type are produced by mixing a well-defined proportion of attenuated IBD virus (**IBDV**) produced in embryonated eggs with specific antibodies produced in specific pathogen free (**SPF**) birds inoculated with IBDV [5]. Recombinant IBDV vaccines use a herpes virus of turkeys (**HVT**) as a vector. The VP2 gene from a donor IBDV is inserted into the genome of the HVT vaccine, which expresses the protein of IBDV as it replicates, thus inducing IBDV protection in the vaccinated animal. Studies demonstrated that HVT did not spread or spread poorly to chickens that had been exposed [6–7], so despite its safe and high efficacy [8], the HVT-vectored IBD vaccines do not have the same potential to produce lateral immunization by horizontal transmission the way IBD live vaccines do. All told, these very different IBD vaccine technologies, when used over time, require constant monitoring of the field challenge due to their differing features and mechanisms of action.

The purpose of this field report is to present the findings of a comprehensive surveillance study of IBDV according to the different vaccination programs in Brazil.

## MATERIALS AND METHODS

### *Sampling Procedures*

In the present study, a total of 246 bursal samples were collected from 2011 to 2014 and submitted to the Zoetis Diagnostic Services Laboratory in Durham, NC, for molecular analysis. All the samples included in this survey were sourced from commercial broiler farms located in Brazil's major poultry producing areas, which were divided as follows: south region (**S**), comprised of the states of Rio Grande do Sul (**RS**), Santa Catarina (**SC**), and Paraná (**PR**); southeast region (**SE**), comprised of the states of São Paulo (**SP**), Minas Gerais (**MG**), and Espirito Santo (**ES**); midwest region (**MW**), comprised of the states of Mato Grosso (**MT**), Mato Grosso do Sul (**MS**), Goiás (**GO**), and Distrito Federal (**DF**); northeast region (**NE**), comprised of the states of Bahia (**BA**), Pernambuco (**PE**), Ceará (**CE**), Sergipe (**SE**), and Pará (**PA**). All farms involved in this study use an integrated system. Sampling and bird management procedures followed the national guidelines for animal care and welfare [9].

Bursal samples were taken predominantly from farms that had been on one of the following vaccination programs for at least 2 grow-out cycles: I. Vector based (HVT+IBD) vaccine, administered exclusively in a single dose at the hatchery level, either subcutaneously or in ovo; II. Immune complex type vaccine (IBDV + bursal derived antibodies), administered exclusively in a single dose at the hatchery level, either subcutaneously or in ovo; III. Conventional live vaccines: freeze-dried vaccines administered exclusively at the farm level via drinking water.

All samples were collected from broiler flocks between 25 and 35 dof age (within 5 d of the previously determined target of 30 d). Actual sampling date and individual flock age for every farm were recorded. Criteria offered for farm selection included recent episode or history of vv IBDV outbreak(s), high mortality or clinical manifestation of disease, inconsistent performance or other evidence of immunosuppression, or reported vaccine failure with partial protection. The average ages for flocks in groups I, II, and III were 30.2, 28.3, and 29.8 d, respectively. At least 5 birds were randomly selected from each poultry house and pooled. Bursal tissue from each bird was directly imprinted onto FTA cards [10].

### *Molecular Assay Description and Procedures*

Detection of IBDV was based on the reverse transcription PCR reaction, followed by amplification of the semi-nested or nested PCR technique of gene regions of the VP2. Each isolate was pooled from one FTA card. Three 3 mm punches per circle were collected from each FTA card. The FTA card punches were lysed in 600 $\mu$l of buffer RLT [11] and incubated for 10 min at room temperature. Six-hundred ul of 70% ethanol were added to the lysis FTA punches. The supernatant was transferred to a RNA column and the ribonucleic acid (**RNA**) was extracted using the RNeasy Mini Kit following the manufacturer's instructions [11]. The extracted RNA was eluted in 50 ul of

Rnase-free water. The RNA product was stored at -20° C until analysis [12]. Real-time (RT)-PCR was performed using a Qiagen One-Step RT-PCR kit following the manufacturer's instructions [11]. Samples were placed in a C1000 thermocycler [13] and RT-PCR was performed using the following parameters: Reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 15 min, denaturation at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for55 s, and final extension at 72°C for 55 seconds. Steps 3 to 5 were repeated for 36 cycles [14]. The amplified sequence of the VP2 was 743 bp. Positive samples were treated with ExoSAP-IT by adding 5 ul of sample to 2 ul of ExoSAP-IT (Affimatrix) and incubated at 37°C for 15 min and then 80°C for 15 minutes. ExoSAP IT treated samples were sent to GENEWIZ, Inc. [15] for sequencing. Amino acid sequences were aligned via the Clustal W method using DNASTAR Lasergene 11 core software [16]. IBD sequences included in the analysis from GenBank [17] were: UK661 (NC004178), Edgar (AY462026), Lukert (AY918948), V877 (AJ586968.1), (AF133904), Del-A (M64285.1), 2512 (DQ355819), STC (D00499), Strain 03–10,681 Canada (EF138990.1), Brandeleiro Brazil 1990 (AY780421), and serotype 2 (U30818.1).

The strains found in this study were classified as vaccines when they were most genetically similar with the Edgar, Lukert, V877, and 2512 IBD sequences, since these are the main vaccine strains used commercially in Brazil. Strains were considered hypervirulent virus when they demonstrated similarity with the UK661 strain, and were considered variant virus when they resembled the Del-E, Del-A, 28–1, 03–10,681 Canada and Brandeleiro Brazil 1990 strains. Percentage of similarity between the isolates and the reference strains were included in front of each isolate in the phylogenetic trees (Figures 1, 2, and 3). The nucleotide sequences for these isolates were submitted to the National Center for Biotechnology Information (**NCBI**) and are available in GenBank across individual accession numbers [17].

Phylogenetic trees were constructed using DNASTAR MegAlign software's neighbor-joining analysis [16]. Bootstrap values were calculated based on 1,000 replicates. Clustal W method was used as a multiple amino acid sequence alignment to create the phylogenetic trees [18].

## RESULTS AND DISCUSSION

### *Demographic Epidemiology*

Table 1 presents the overall findings, grouped by geographical area. A total of 75 IBDV strains was positively identified. The overall recovery rate was 30%. All regions showed similar proportions of positive samples for IBDV. The genetic analysis of positive samples, classified into vaccine virus (classic strains) or field virus (vvIBDV or variant strains), was similar among the different regions studied. In fact, vvIBDV and variant strains were encountered at similar frequencies in all regions where they were found, except in the MW, where only one strain of vvIBDV was detected [19]. This fact may be related to the low number of samples analyzed in this region. The region with a slightly higher incidence of a positive field virus (variant or vvIBDV) was the S, where most Brazilian poultry production is located. This finding is in agreement with the fact that general control of diseases including IBD is more difficult in areas with a higher population density of birds [20].

In contrast, in the 1990s, geographically isolated states in the west and northwest regions of the United States with lower population densities demonstrated the reverse, with 94% of field isolates categorized as classical strains [21]. Recent references describe predominance of variant virus among field isolates in the United States [22]. These epidemiological findings support the idea that the differences between geographical regions and over time are related to the production system used, including the vaccination program adopted [23].

Previous RT-PCR results for Brazilian regions with large broiler production suggested that domestic IBDV isolates originated from the Netherlands, especially in the case of very virulent strains (vvIBDV), and in the case of variants, these isolates seem to have come from the United States via animals imported with subclinical IBD [24]. The source of vvIBDV was suspected to be imported Dutch tulip bulbs that

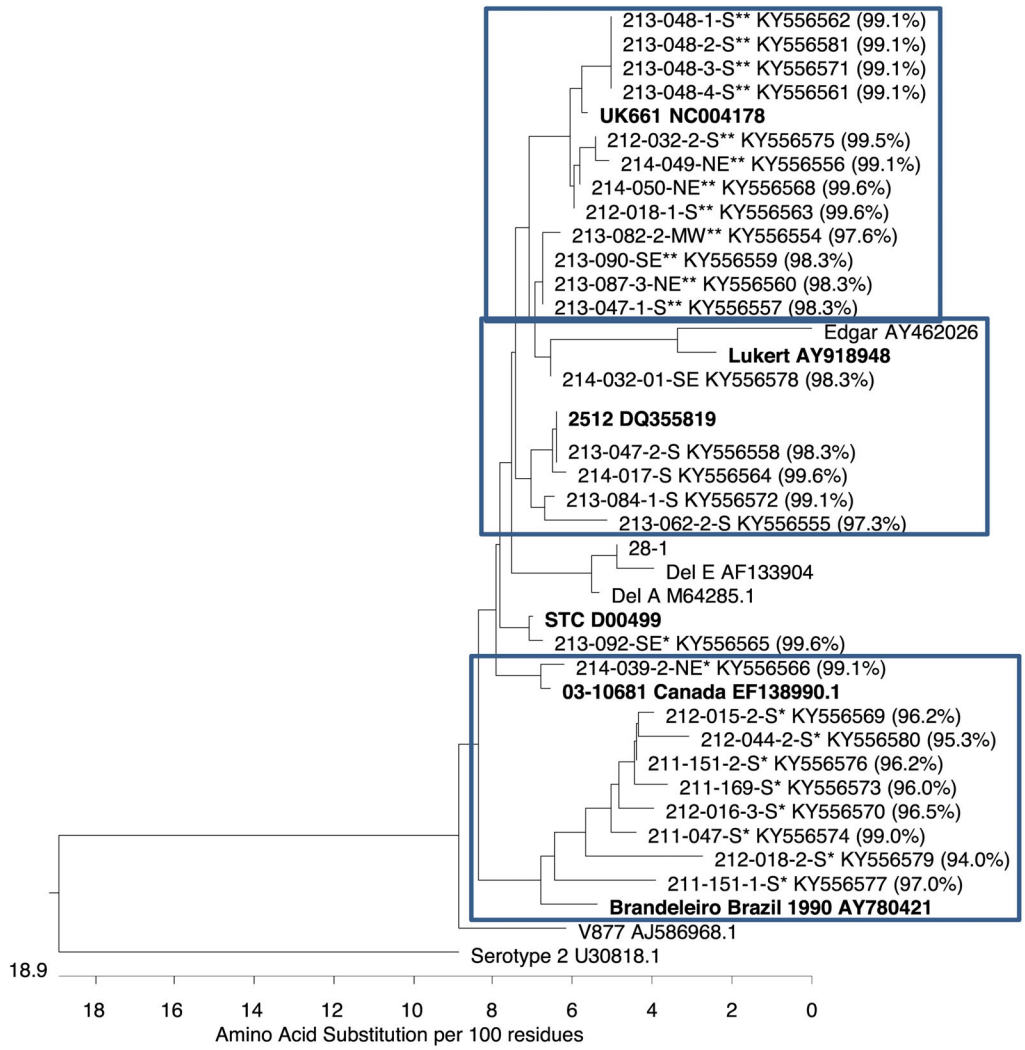


**Figure 1.** The ML phylogenetic tree generated using the VP2 in amino acids hypervariable region of IBD viruses detected in commercial poultry that received a vectored IBD vaccination program. *variant virus, **vvIBDV. S = South, SE = Southeast, MW = Midwest, NE = Northeast. The isolates can be searched with Genbank accession numbers.

had been fertilized with contaminated chicken manure [25]. Classical IBDV, including vvIBDV, is reported in molecular surveys in European territory [26].

### *IBDV Detection and Classification*

Table 2 summarizes the number of IBDV strains recovered from samples according to the 3 groups of primary vaccination strategies adopted by the participating farms. In each category, the total number of positive samples was further classified into presumably field or vaccine virus, based on the respective clinical history of each farm, the nucleotide sequences of the viruses, and the phylogenetic clustering information of individual strains, as presented in the molecular assay description.

### *Phylogenetic Clustering*

Data related to virus characterization and phylogenetic analyses are presented below. Molecular patterns of the major vaccine strains

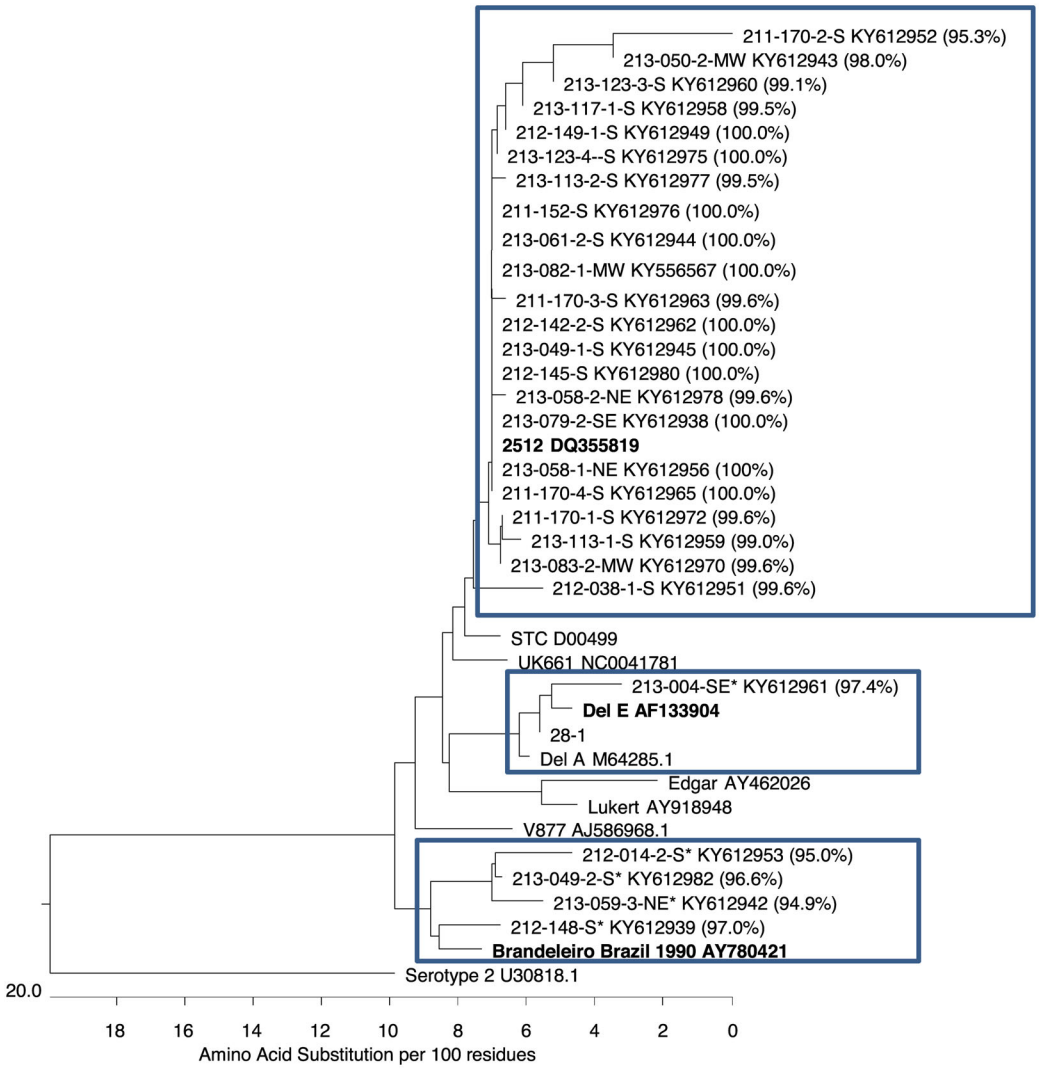


**Figure 2.** The ML phylogenetic tree was generated using the VP2 in amino acid hypervariable region of IBD viruses detected in commercial poultry that received an immune complex vaccination program. *variant virus, **vvIBDV. S = South, SE = Southeast, MW = Midwest, NE = Northeast. The isolates can be searched with Genbank accession numbers.

that are commercially available in Brazil have been included in each phylogenetic tree for reference.

In the vectored vaccination strategy group, almost two-thirds of the samples (50/77, 65%) yielded negative PCR results. Theoretically, immunity is primarily elicited by the VP2 protein inserted in the HVT construct, and therefore no live or replicate IBDV particles are introduced into the birds during vaccination. Consequently, the negative results are expected for birds vaccinated with this technology, since there is no replication of the vaccine strain in the parenchyma of the bursa of Fabricius. However, analysis of the sequencing results from positive IBDV samples (27/77, 35%) sourced from farms using only vector HVT + IBD vaccine indicates a prevalent molecular pattern that demonstrates close similarities with the reference isolate UK 661 (Figure 1), originally described as vv strain (vvIBDV) [27]. The phylogenetic tree analysis also demonstrates the presence of variant IBDV, as well as classic IBD viruses.

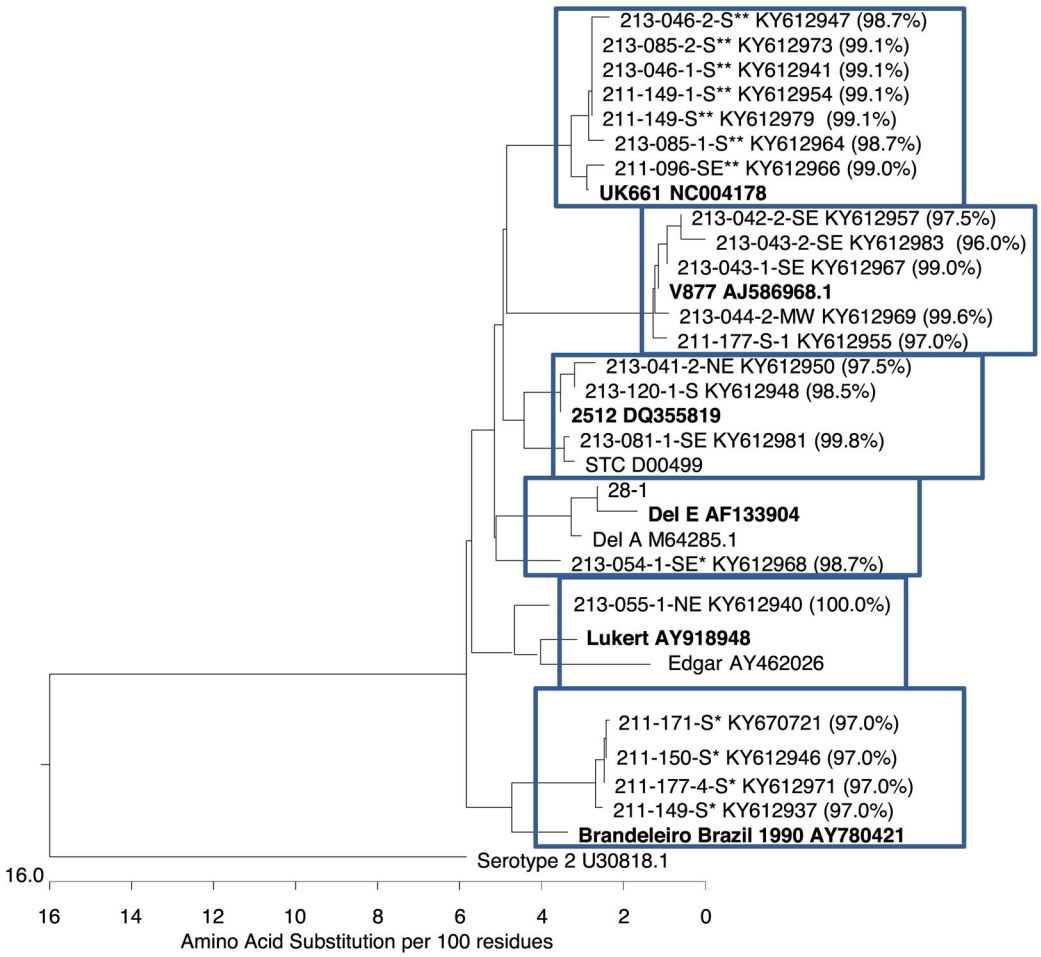


**Figure 3.** The ML phylogenetic tree generated using the VP2 in amino acids hypervariable region of IBD viruses detected in commercial poultry that received conventional field vaccination programs. *variant virus, **vvIBDV. S = South, SE = Southeast, MW = Midwest, NE = Northeast. The isolates can be searched with Genbank accession numbers.

**Table 1.** Results of IBDV analysis according to the region of Brazil studied.

| | | Total samples | | Positive samples | | |
|---|---|---|---|---|---|---|
| Geographical region | Total samples | Negative | Positive (vaccine or field virus) | Vaccine | vvIBDV | Variant strain |
| **South (S)** | 153 | 103 (67%) | 50 (33%) | 22 (44%) | 13 (26%) | 15 (30%) |
| **Southeast (SE)** | 46 | 35 (76%) | 11 (24%) | 6 (55%) | 2 (18%) | 3 (27%) |
| **Midwest (MW)** | 12 | 7 (58%) | 5 (42%) | 4 (80%) | 1 (20%) | 0 (0%) |
| **Northeast (NE)** | 35 | 26 (74%) | 9 (26%) | 4 (45%) | 3 (33%) | 2 (22%) |
| **Total** | **246** | **171 (70%)** | **75 (30%)** | **36 (48%)** | **19 (25%)** | **20 (27%)** |

**Table 2.** Results of IBDV analysis according to vaccination programs.

| | | Total samples | | Positive samples | | |
|---|---|---|---|---|---|---|
| Vaccination strategy | Total samples | Negative | Positive (vaccine or field virus) | Vaccine virus | vvIBDV | Variant strain |
| **Vector based** | 77 | 50 (65%) | 27 (35%) | 5 (19%) | 12 (44%) | 10 (37%) |
| **Immuno-complex** | 105 | 78 (74%) | 27 (26%) | 22 (81%) | 0 (0%) | 5 (19%) |
| **Conventional** | 64 | 43 (67%) | 21 (33%) | 9 (43%) | 7 (33%) | 5 (24%) |
| **Total** | **246** | **171 (70%)** | **75 (30%)** | **36 (48%)** | **19 (25%)** | **20 (27%)** |

In Brazil, a classic IBDV genetically related to the vv pathotype originally described by Di Fabio et al. [28] has been found in bursas from farms experiencing outbreaks of acute mortality. Ikuta et al. [29] also reported presence of vvIBDV by molecular characterization demonstrating field challenge by this type of virus in Brazilian poultry.

In our study, we isolated vvIBDV from 12 of 27 IBDV positive samples from flocks vaccinated with vectored vaccines, as well as in over 16% (12/77) of all samples from flocks vaccinated with vectored vaccines (Table 2). According to Müller et al, "... the vectored vaccines may pave the way to a sustained and successful IBD prevention and control regimen in the near future. It has been speculated, however, that in the field it may be difficult to maintain high efficacy" [30].

In the group of samples sourced from farms primarily using the immune complex type of vaccines (IBDV+BDA), the majority of positively identified IBDV (22/27) were similar to the reference Winterfield 2512 strain, which is the antigen included in the formulation of the main products in this category, such as Transmune® (CEVA) and Bursaplex® (Zoetis), both of which are commercially available in most international markets.

The sequence analysis of the remaining 5/27 IBDV positively tested samples (Figure 2), which cluster in the lower portion of the phylogenetic tree, indicated that these strains are variant IBDV with a potential immunosuppressive effect. These findings suggest that under specific circumstances, field-resident IBDV strains are still capable of infecting flocks by at least 25 to 35 d of age, despite the active immunity elicited by hatchery vaccination. The ability of these field viruses to break through this immunity and infect young flocks may depend on their virulence and antigenicity. The vvIBDV have been shown to break through maternal immunity to IBD because they are highly invasive [31]. Antigenic drift also has contributed to the evolution of viruses that can infect chicks when immunity to IBD is relatively high [32]. Infections during this window of opportunity may, depending on the characteristics of the strain involved, result in immunosuppression and secondary infections. However, under the specific conditions of this study it was not possible to demonstrate a direct correlation between the detection of viruses in bursal tissue and potential negative impact on flock performance.

The Maximum Likelihood (**ML**) phylogenetic tree (Figure 3) maps the occurrence of IBDV strains recovered from broiler flocks in which the main vaccination strategy was administration of conventional live vaccines via drinking water. Of the total of 64 FTA cards, 21 tested positive (33%) for IBDV. Of these, 9 were characterized as vaccine strains by cross evaluating the PCR molecular assay results for each individual case with the profile database of the main vaccines commercially available in Brazil. In general, live vaccination in this group of farms was performed using intermediate (i.e., Lukert) or intermediate-plus (i.e., 2512 and V877) strains.

Regardless of the choice of a particular strain or vendor from any of the suppliers of live IBD vaccines in Brazil, almost half of the positive isolations in this group (9/21 or 43%) were nearly perfect genetic matches for the respective commercial product in use. The remaining 12 IBDV positive samples (57%) fell fairly evenly into the 2 aforementioned field IBDV categories: vvIBDV (7/12 or 58%) and variant virus (5/12 or 42%).

Conventional live vaccines are based on classic IBDV strains. In this survey, there was no high-level protection against the field challenge, as 11% of all tested samples were positive for vvIBDV. The effectiveness of conventional live attenuated vaccines is often hindered, not by their quality or safety spectrum, but by either maternal antibody interference or the challenges in properly executing drinking water vaccination in the field [33]. This vaccination process must account for the particular nuances on each farm, such as the structure of the hydraulic system, skill of the vaccination team, water quality, and number of birds, among other factors [34].

## CONCLUSIONS AND APPLICATIONS

1. Of the 246 broiler flocks analyzed, we identified 75 IBDV positive flocks (30%), with approximately half of the positive samples classified as field strains and the other half compatible with the vaccine genotype based

on PCR analysis of the hypervariable region of IBDV VP2.

2. Field strains of IBDV were found among the different regions analyzed in this epidemiological survey. The field challenge seems to be homogeneously present in Brazil and divided almost equally between vvIBDV and variant strains.
3. The high percentage of field virus either by classical or variant strain demonstrates that even where birds are subjected to the immune prophylactic scheme with administration of live attenuated vaccines, antigen-antibody complex, or recombinant IBDV, there is a very dynamic landscape where the field viruses seek to evolve and overcome the immune barrier. There is no perfect prophylactic scheme to cover all field challenges.
4. Under the evaluated conditions, there was a high detection rate (29%) of field viruses in flocks vaccinated with vectored vaccine, which demonstrates the ability of the field virus to occupy space in the bursa of Fabricius and consequently in the environment. This also occurred when other prophylactic schemes were used, albeit at lower rates using live attenuated (19%) or antigen-antibody complex (5%).
5. The various immune strategies must be monitored using field surveillance to measure their effects on the field challenge in a particular region. There is no perfect vaccine category for managing all situations. The various strains of field IBDV are continuously being selected and looking for ways to persist in the birds and the environment.
6. Based on the high detection rate of the field virus in vaccinated flocks, inoculation studies should be carried out in SPF birds with these strains, particularly variants, in order to understand the pathogenic and immunosuppressive potential of these viruses.
7. Future studies on the epidemiology of IBDV associated with different vaccination programs should include data related to productivity, mortality, clinical signs, and lesions as well as condemnations in broiler flocks to address the performance of IBDV vaccines.

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