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Control of Field Infectious Bronchitis Virus Genotype GI-23 (Variant 2) Using Combined Heterologous Vaccine Genotype Strains GI-13 (1/96) and GI-1 (H120)

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ABSTRACT

Infectious bronchitis (IB) is a disease with significant economic impacts both on the costs of control strategies and on productive losses. Various vaccination protocols are applied, depending on homologous or heterologous protection against IB and finding the optimal balance between costs and benefits as a choice by a responsible veterinarian. The current case study aimed to demonstrate the efficacy of a heterologous vaccine combination against field IBV GI-23 (Variant 2) infection when vaccination quality was properly monitored. Two groups, each consisting of six flocks, were examined before and after improving the quality of IB vaccine application in the hatchery. These groups were vaccinated with H-120 and 1/96 vaccine strains for heterologous protection. The study involved field visits, necropsies, serology via ELISA, and oropharyngeal sampling for RT-PCR follow-up activities. Moreover, performance parameters including average body weight, feed conversion ratio, and 7 days plus total mortality were analyzed at the end of the production cycle at 40-45 days of age. Results indicated that the group with enhanced vaccination quality in the hatchery exhibited a significant decrease in IBV titers and an absence of IBV GI-23 field infection. Additionally, there was an improvement in performance data in terms of average body weight, FCR and total mortality. Hatchery vaccination proved to be more controllable and practical compared to traditional on-farm vaccination, ensuring better control of the vaccination process and massive coverage of the farm population.

Keywords: Heterologous protection, Infectious bronchitis, Vaccine strain 1/96, Variant 2

INTRODUCTION

Infectious bronchitis (IB) is a disease that causes major economic losses for the poultry industry worldwide. It has been listed as one of the most harmful diseases for livestock (World Bank; TAFS Forum, 2011). IB is a highly transmittable viral disease caused by an avian coronavirus whose taxonomy has previously been fully described (Coronaviridae family, Orthocoronavirinae subfamily, Gammacoronavirus Genus, and Igacovirus subgenus; Lefkowitz et al., 2018). The genome of IBV is a single-stranded positive-sense RNA and its properties have been extensively analyzed (Boursnell et al., 1987). The IBV's high substitution and recombination rate is similar to other single-stranded RNA viruses (Duffy et al., 2008; Simon-Loriere & Holmes, 2011) and it can lead to the emergence of several genotypes and lineages over time (Valastro et al., 2016). In terms of prevention and population dynamics, live attenuated vaccines are the most effective ones (Jordan, 2017). The most used IB vaccines are live attenuated vaccines, which can mimic the natural infection process of the field viruses with minor post-vaccination reactions and proven efficacy by inducing strong humoral, cellular, and local immunity against closely related strains (Bande, 2015; Bhuiyan et al., 2021). Live vaccines have demonstrated efficacy in widening the conferred protection by combining unrelated IBV vaccine strains to protect against other IBV clusters, an effect which is known as heterologous protection (Cook et al., 1999). Protection with homologous vaccines has also been introduced but their applicability is limited due to interpretation and differentiation of field strains (Legnardi et al., 2022a). Having stated that, several experiments have been performed to explain the mechanism of heterologous or cross-protection vaccination such as assessing kinetics (Tucciarone et al., 2018) and promoting cross-protection

levels by different vaccines through several experimental trials (Franzo et al., 2016; Legnardi et al., 2022b). In such trials, different administration routes and vaccination timings were studied using various assessment methods like quantification of the viral load of the vaccine strains from tracheal swabs (Jackwood, 2009), ciliostasis observation, and challenge virus detection (Tatar-Kis et al., 2014). Among the numerous IBV variant lineages, Genotype I-23 (GI-23) is the most prevalent in the Middle East and Persian Gulf countries, Western Africa, Southern Africa, Turkey, and Eastern Europe, with sporadic detections in Germany and Benelux (Houta et al., 2021). GI-23 was characterized macroscopically and it was found to induce depression, huddling, respiratory symptoms, and diarrhea with a 30% mortality rate 6 days post-infection (dpi) in Specific Pathogen Free (SPF) broiler chickens (Lisowska et al., 2021). Moreover, in regions affected by GI-23, increased virulence and co-infections with other pathogens, such as low-pathogenic avian influenza H9N2, *Escherichia coli*, or *Mycoplasma*, have been reported in commercial poultry farms (Samy and Naguib, 2018). The present case study aimed to demonstrate, through Real-World Evidence analysis, the efficacy of a heterologous vaccine combination against field IBV GI-23 (Variant 2) infection.

MATERIALS AND METHODS

Ethical approval

Since the sampling of the broilers was done during common commercial activities in the farm regulated by national and international laws, ethical review and approval were waived for this study.

Experimental design

Six commercial Ross 308 broiler flocks of 20,000 to 30,000 broilers each with *ad libitum* access to feed and water were investigated in the South-Eastern region of Romania (Ialomița county) from April 2022 to March 2023. Upon the farmer's call, the main symptoms observed in the affected flocks included the general lack of performance, increased mortality, nephritis, watery diarrhea, and wet litter. The diseases including IBV, infectious bursal disease (IBD), Avian rhinotracheitis (ART), Newcastle disease (NDV), *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS) were investigated for differential diagnosis using either RT-PCR or serology (Table 1). Due to local constraints, no access to the hatchery premises was initially allowed; therefore, the quality of application of the IBV vaccine was not assessed until the final investigation stage, when vaccine preparation and application were inspected following Bureau Veritas® Ceva C.H.I.C.K. program recommendations (Franzo et al., 2019). Field, follow-up activities, such as necropsy, serology and RT-PCR, were initiated from the first day of investigation. As a result, two different groups were proposed based on the quality of IB vaccine application in the hatchery: 1) Flocks vaccinated before inspection (group A); 2) Flocks vaccinated after inspection by Bureau Veritas® Ceva C.H.I.C.K. program (group B).

Serology

For both groups, individual blood samples from 100 broilers were collected from all flocks for ELISA analysis by wing puncture from a branchial vein (Kelly and Alworth, 2013) using a small needle, in accordance with all animal welfare protocols between 39 to 42 days of age (doa). After collecting approximately 2 ml of blood from the wing (according to the Biochek® ELISA Kit manufacturers' recommendations, Biochek, Netherlands), individual sera were collected, labeled, and sent for further analysis (Synevovet Str. Industriilor, Nr. 25, comuna Chiajna judetul Ilfov, 077040 Romania).

RT-PCR

Individual oropharyngeal samples for RT-PCR (Tucciarone et al., 2018) were printed on Qiagen® indicating FTA cards (Qiagen, Hulsterweg 82, 5912 PL Venlo, The Netherlands) and sent for further processing to a third-party diagnostics laboratory (De Gezondheidsdienst voor Dieren, Postbus 9, 7400 AA Deventer, The Netherlands) (Table 1).

Table 1. Investigated diseases, methods of investigation, and age of sampling per house in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccines

House	Date	Investigated diseases	Age of sampling (doa)	Serology (n)	RT-PCR (n)
9	19-04-2022	IBV	41	Y (20)	N
8	09-08-2022	IBV, IBD, NDV	42	Y (20)	N
7	03-10-2022	NDV, IBV	42	Y (20)	N
3	22-11-2022	NDV, IBD, IBV, MG, MS, ART	39	Y (20)	Y (4)
3	19-12-2022	IBV	6	N	Y (8)
3	19-01-2023	NDV, IBD, IBV	41	Y (20)	Y (4)

Y: Yes, N: No; Doa: Days of age; n: Number of samples.

Table 2. Vaccination program during the present study.

Vaccine	Manufacturer	Disease	Age (doa)	Site	Route
Cevac® Broiler ND K	Ceva Santé Animale	Newcastle Disease	1	Hatchery	Subcutaneous
Cevac® Transmune IBD		Infectious Bursal Disease	1	Hatchery	Subcutaneous
Cevac® Vitabron L		Infectious Bronchitis	1	Hatchery	Spray
Cevac® IBird™		Infectious Bronchitis	1	Hatchery	Spray
Cevac® New L		Newcastle Disease	10	Farm	Spray
Cevac® New L		Newcastle Disease	20	Farm	Drinking water

doa: Days of age.

Vaccination program

The broiler prophylactic program, techniques, and machinery used for vaccination remained intact throughout the investigational period. The vaccination program included IBD, ND, and IB live vaccines applied both in the hatchery and on the farm. The machine used for IB spray vaccination at day 1 was an Ecat® Spray Cabinet (Ecat-ID, France). Subcutaneous vaccination was done with a Dovac® double V2 injector (Ceva, 2024; Table 2).

Performance parameters

The performance of 9 houses from 6 flocks during 4 consecutive cycles was obtained and correlated with the previous analyses. The collected data included the day of chick placement, age at slaughter, average body weight, Feed Conversion Ratio (FCR), mortality at 7 days of age, and total mortality.

Statistical analysis

Statistical visualizations and tests were performed using Python, v3.10.6.2022. Non-parametrical Wilcoxon-Mann-Whitney (test U of Mann-Whitney) with an alpha-risk at 5% was used, considering the relatively low-performance data points (18 in Group A vs. 18 in Group B).

RESULTS

Serology

The analysis of the titers during the investigational period (April 2022 to March 2023) indicated a wide span of individual titers (Figure 1).

According to the ELISA Kit supplier's interpretation, above 3000 units is the threshold at which field challenges can be considered. The range of titers increased widely above 3000, reaching up to 14000 units in the examined flocks. In addition, a significant difference ($p < 0.05$) in the titers between houses was observed (Figure 2). As stated above, groups A and B were selected according to the maintenance state of the hatchery sprayer cabinet. When comparing the titers of these two groups, a strong significant difference was detected between them, with the group before auditing in the hatchery having significantly higher titers (Figure 3). When analyzing the individual titers in the two groups, most of the individual titers in group A were above that limit (n = 53; 66,3%). In group B, only 4 out of 20 broiler chickens (20%) had titers above the 3000-unit limit (Figure 4).

RT-PCR

The number of RT-PCR results was not significant to compare groups using statistical analysis. RT-PCR yielded positive results for GI-23 (Variant 2) in group A and positive results for GI-13 (1/96) in group B at 6 and 41 days of age (Table 3).

Performance

Performance data of 36 flocks was obtained and analyzed (18 versus 18, Table 4). The null hypothesis (H0) was based on the absence of difference between the two groups, whereas the alternative hypothesis (Ha or H1) was based on a difference between the two groups. Due to the low number of data points, it was not possible to find a statistically significant difference between groups A and B in density, age at slaughter, total mortality, Average Body Weight, and FCR (Figures 5 and Table 5).

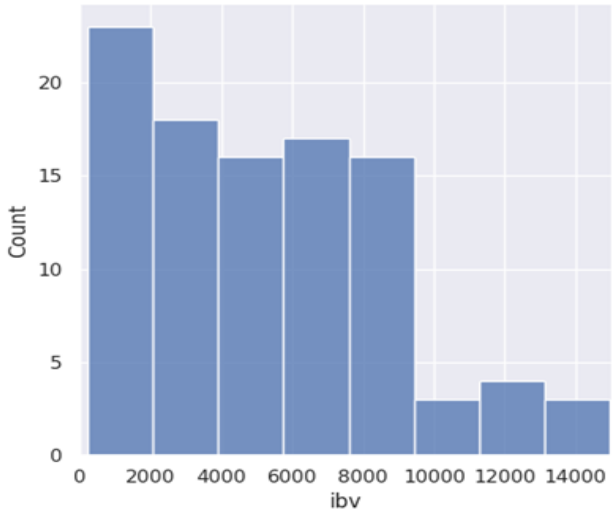

Figure 1. Distribution of ELISA Infectious Bronchitis Virus titers (n= 100) in the flocks (n= 6); in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains. Serology was analyzed by Biochek® ELISA IBV kit

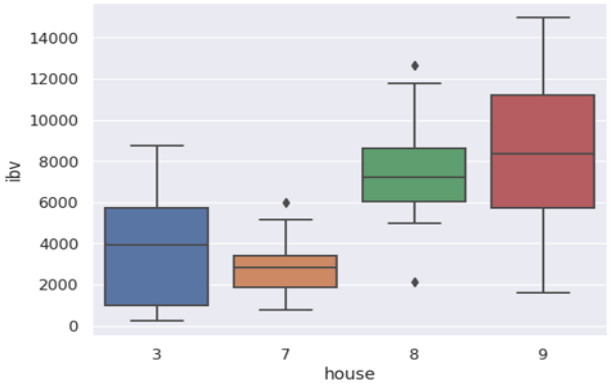

Figure 2. Distribution of ELISA Infectious Bronchitis Virus titers (n=100) in the investigated houses; in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains. Biochek® ELISA IBV kit; (test U of Mann-Whitney; $p < 0.05$)

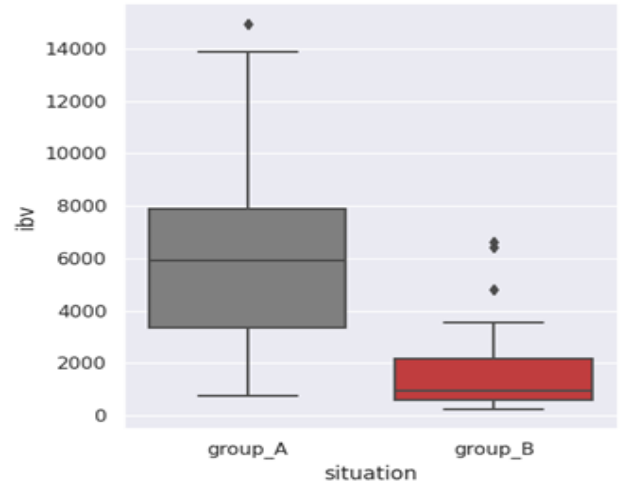

Figure 3. Distribution of ELISA Infectious Bronchitis Virus titers per group before and after auditing in the hatchery (n = 100); in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains. Biochek® ELISA IBV kit; (test U of Mann-Whitney; $p < 0.05$)

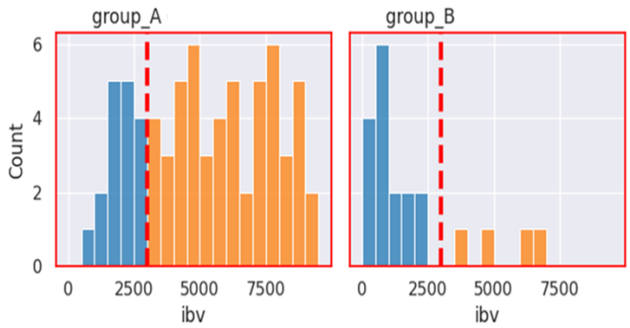

Figure 4. Distribution of individual ELISA Infectious Bronchitis Virus titers per group before auditing (group A) and after auditing (group B) in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine Genotype GI-13 (1/96) and GI-1 (H120) strains. n = 80 in Group A; n = 20 in group B; Challenge limit = 3000 units.

Table 3. Age of sampling, number, and type of sample in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains

House	Group	Date	Investigated Disease	Age of sampling (doa)	RT-PCR sample type (n)	Results
3	A	22-11-2022	IBV	39	Oronasal (1) Caecal tonsils (1) Kidney (2)	GI-23 (Variant 2)
3	B	15-12-2022	IBV	6	Oropharyngeal swabs (8)	GI-13 (1/96)
3	B	19-01-2023	IBV	41	Caecal tonsils (4)	GI-13 (1/96)

n: Number of samples; doa: Days of age

Table 4. Performance of group A (before auditing) and group B (after auditing) in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains

House	Placement date	Group	Age at slaughter	ABW	FCR	Mortality 0-7 d (%)	Total mortality (%)	Density (m^2)
1	22/08/2022	Group A	42	2853	1,63	0,32	2,32	21,20
2	18/08/2022	Group A	42	2830	1,62	0,35	2,61	20,61
3	18/08/2022	Group A	43	2932	1,61	0,35	2,75	20,61
4	22/08/2022	Group A	42	2845	1,61	0,43	2,91	21,20
5	22/08/2022	Group A	43	3040	1,62	0,52	3,03	21,20
6	22/08/2022	Group A	42	2808	1,63	0,50	2,65	21,20
7	22/08/2022	Group A	43	2869	1,63	0,54	2,54	21,20
8	22/08/2022	Group A	42	2831	1,64	0,60	2,41	21,20
9	22/08/2022	Group A	43	2855	1,65	0,58	2,68	21,20
1	14/10/2022	Group A	42	2890	1,61	0,41	2,39	20,25
2	14/10/2022	Group A	41	2649	1,66	0,42	4,53	20,25
3	14/10/2022	Group A	41	2646	1,65	0,51	4,71	20,25
4	14/10/2022	Group A	42	2880	1,61	0,51	2,28	20,25
5	17/10/2022	Group A	42	2782	1,59	0,26	2,69	20,86
6	17/10/2022	Group A	42	2802	1,59	0,29	2,68	20,86
7	17/10/2022	Group A	43	2833	1,61	0,29	3,13	20,86
8	17/10/2022	Group A	43	2843	1,62	0,27	2,81	20,86
9	17/10/2022	Group A	43	2732	1,61	0,45	3,43	20,86
1	09/12/2022	Group B	41	2769	1,58	0,46	2,95	20,43
2	09/12/2022	Group B	40	2702	1,56	0,51	2,86	20,43
3	09/12/2022	Group B	41	2781	1,59	0,45	2,90	20,43
4	09/12/2022	Group B	41	2746	1,59	0,40	3,11	20,43
5	09/12/2022	Group B	41	2855	1,59	0,44	3,00	20,43
6	12/12/2022	Group B	44	3026	1,60	0,33	2,81	21,03
7	12/12/2022	Group B	43	2909	1,60	0,32	2,57	21,03
8	12/12/2022	Group B	44	2888	1,60	0,38	2,64	21,03
9	12/12/2022	Group B	43	2775	1,57	0,32	2,81	21,03
1	03/02/2023	Group B	45	3122	1,64	0,52	2,67	20,79
2	03/02/2023	Group B	41	2810	1,62	0,51	2,73	20,79
3	03/02/2023	Group B	42	2855	1,63	0,48	2,63	20,79
4	03/02/2023	Group B	42	2826	1,62	0,58	2,85	20,79
5	03/02/2023	Group B	41	2651	1,62	0,25	1,99	20,79
6	06/02/2023	Group B	43	2855	1,63	0,35	2,26	21,33
7	06/02/2023	Group B	42	2835	1,62	0,61	2,45	21,33
8	06/02/2023	Group B	42	2927	1,62	0,30	1,97	21,33
9	06/02/2023	Group B	43	2868	1,63	0,51	3,03	21,33

ABW: Average body weight at slaughter; FCR: Feed conversion rate; doa: Days of age.

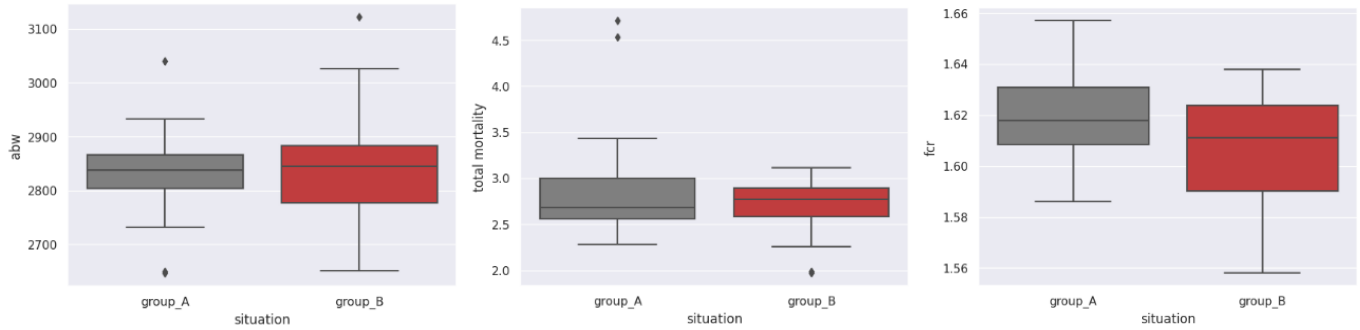

Figure 5. Differences in performance between groups A and B in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotypes GI-13 (1/96) and GI-1 (H120) strains. ABW: Average body weight at slaughter; FCR: Feed conversion rate (test U of Mann-Whitney; $p < 0.05$)

Table 5. Average body weight, FCR, and mortality in control of field infectious bronchitis virus genotype GI-23 (variant 2) with the combined use of two heterologous vaccine genotype GI-13 (1/96) and GI-1 (H120) strains

KPI	Group A: average	Group B: average	Group A: std	Group B: std	p-value
Age at slaughter	42.277778	42.16667	0.669113	1.339447	0.562474
Density (birds/m^2)	20.828889	20.863333	0.376140	0.338509	0.910984
Average body weight	2828.888889	2844.444444	91.861077	110.434022	0.824615
FCR	1.620778	1.606167	0.019907	0.023183	0.136859
Mortality 0-7 d	0.422222	0.428889	0.110536	0.102721	0.849134
Total mortality	2.919444	2.679444	0.683662	0.330285	0.727721

KPI: Key performance indicators; std: Standard deviation.

DISCUSSION

In this field investigation, a thorough analysis of all the predisposing factors influencing disease occurrence was undertaken. However, as previously explained, access to the hatchery was only granted after the analysis of the first three production cycles in 2022. Therefore, the quality of the vaccine application was audited only after that time point. This factor initially compromised the implementation of all necessary mechanisms required to find out the root cause of the issue.

In terms of serology, a wide distribution of titers was observed in the investigated houses. Geometric Mean Titer values indicated a representative difference ($p < 0.05$) between both groups before and after the investigation. However, the consideration that individual IBV-ELISA titers reflect challenging situations on the farm in a more specific way was eminent. The method of considering 3000 ELISA units as the threshold for flocks that suffer IBV-variant challenge was within the recommendations of the ELISA kit manufacturer.

In this sense, the range of individual ELISA-IBV titers spanned from 0 to 15000 ELISA units, with the majority of samples above the 3000 limit, indicating a disease challenge aligned with previous experiences (Cortés et al., 2022). Indeed, the titer differences between groups A and B indicated that this might have been occurring before the failure in the vaccination technique was discovered in the hatchery. The importance of audit control on vaccination quality has been well established previously by Franzo et al. (2019). Once the issue was addressed, the ELISA-IBV titers dropped dramatically, falling further below the 3000-unit threshold. This indicated that GI-23 was under control and confirmed the initial hypothesis and approach to individual serological analysis.

As a confirmation of the previous serological findings, validation of IBV control through RT-PCR was recommended. RT-PCR confirmed GI-23 (Variant 2) clearance in December 2022. The first confirmation of strain GI-13 (1/96) replication was observed at 6 doa via oropharyngeal swabs. This method is an early, useful, and welfare-friendly procedure that addresses vaccination processes directly at the farm level. Final confirmation was achieved via RT-PCR analysis of the caecal tonsils at 41 doa, demonstrating that GI-13 (1/96) outcompeted the GI-23 (Variant 2) field strain detected in the previous flock in November 2022. This confirms the results of previous research works that propose heterologous cross-protection as the only mechanism to control IBV population and genetic drift worldwide, either by direct competitive exclusion or by a strong local immune response that inhibits the replication of the wild IBV strain in the immunized broilers (Franzo et al., 2016; Lisowska et al., 2021). Additionally, heterologous vaccination is not proven to induce actively the escape of wild IBV populations from vaccine-induced immunity over time (Vermeulen et al., 2023). The occurrence of subpopulations within IBV GI-23-based vaccines and the variability featuring different production batches which complicates the differentiation between field and vaccine-derived strains based on sequence analysis alone has also been demonstrated by Legnardi et al. (2022a), thereby presenting another challenge in need of solutions. Generally, IB is accompanied by secondary pathogens that enhance the pathogenicity of the virus and its effects on condemnation rates (Assayag Júnior et al., 2012; Linares et al., 2017). The latter observation was also reflected along with a general impact on performance. However, despite clear trends observed after the improvement of the vaccination process (e.g. mortality and FCR improvement), the number of observations was insufficient to establish a statistically representative difference.

CONCLUSION

Real World Evidence comprises tangible data that enables veterinary professionals to make swift and informed decisions based on well-analyzed field data. In this study, the analysis of field and diagnostic data confirmed that the combination of GI-1 (Mass) and GI-13 (1/96) at day 1 in the hatchery is a highly effective mechanism to protect against the increasing presence of GI-23 (Variant 2) in parts of the world where it is prevalent, making it unnecessary to use a homologous GI-23 vaccine where a Differentiating Infected from Vaccinated Animals (DIVA) strategy is not feasible. Vaccinating in the hatchery is easier to control diseases and more practical to apply than traditional on-farm application. It ensures improving the control of the vaccination process and provides extensive coverage of the farm population, allowing effective protection against several heterologous IBV field strains.

DECLARATIONS

Authors' contributions

Mirel Enache and Mihai Pirvulet contributed to the study design, investigation, and accuracy of the data. Mathilde Lecoupeur, Higor Cotta, Guillermo Gonzalez Garcia, and Konstantinos Koutoulis participated in investigation activities, analysis of the data, writing, reviewing, and editing of the manuscript. All authors checked and approved the final version of the manuscript.

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Ethical considerations

The authors confirm that all contributors to this study have reviewed and submitted the manuscript to this journal for the first time.

Availability of data and materials

The original information presented in the study is included in this article. More data can be requested from the corresponding authors.

Conflict of interests

The authors declare that no conflicts of interest exist.

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