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Original Article

Evaluation of the Performance of a New Pentavalent Vaccine in Poultry

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ABSTRACT

Viral inactivated vaccines play a fundamental role in animal heath, both for individual protection and for the induction of antibodies that will be passively transmitted to the progeny. Depending on the field challenges, it is eventually necessary to combine more than one etiology. Inactivated polyvalent vaccines with appropriate combinations to ensure adequate and balanced responses may induce a satisfactory and long-lasting immune response. This study aimed to evaluate the antibody responses of a new oil-based inactivated pentavalent viral vaccine for intramuscular administration containing a newly developed immunomodulator, and compare it with conventional vaccines through antibody responses to the same antigens by ELISA in successive weeks after administration. Mortality rate, weight gain, egg production and hatching were also determined to evaluate safety. The results obtained revealed significant differences (p<0.05) between the group that received the new vaccine and the control group, presenting persistent and long-lasting IgG (IgY) antibodies in specific pathogen free (SPF) chickens for 146 days. In addition, there was a statistically significant (p<0.05) difference in the serological response of the new pentavalent vaccine in commercial poultry in relation to the monovalent commercial vaccines for infectious bronchitis, avian metapneumovirus, Newcastle disease, Gumboro, and reovirus fractions. There was no change in the productive parameters evaluated when compared to the conventional vaccine or the control.

INTRODUCTION

Vaccination of breeder hens with inactivated polyvalent viral vaccines is widely used in industrial poultry farming, as it results in two important benefits. It both contributes to the protection of the poultry due to the high levels of circulating antibodies generated, while also participating in the production and transfer of part of this immunity to progeny (maternal antibodies) (Marangon & Busani, 2007).

Due to increases in field challenges and to produce a more complete maternal immunity, Brazilian breeder farmers have adopted broad immunoprophylactic programs (Gomes, 2022). Thus, they make use of combinations of inactivated polyvalent vaccines to carry out the desired immunoprophylactic program and achieve a satisfactory duration of immunity, leading to the need for multiple injectable administrations. Such application is usually performed intramuscularly and, in cases of double application, each side of the breast muscle of the poultry receives an injectable application. In addition to the labor intense activity of individual handling of poultry, it is extremely common for the injectable vaccine to produce lesions at the application site, causing stress to the birds. Thus, the concentration of several antigens in the

same product is desirable, as it potentially reduces the multiple administrations of vaccines by intramuscular route (Droual *et al.*, 1990).

Vaccines emulsified in oily adjuvants enhance and standardize the humoral response previously induced by live vaccines, thus reducing the workforce and stress resulting from frequent vaccinations (Schijns, 2000). In an ideal inactivated vaccine, an attempt is made to satisfactorily combine immunogenicity and harmlessness. This constant search for a balance between the ability to induce a solid immune response and the reduction or absence of adverse reactions has resulted in the development of new technologies for the production, formulation, emulsification, and enhancement of the immune response with the addition of immunomodulating components (Salem & Weiner, 2009). The addition of immunomodulators to vaccines can maximize their effectiveness, especially when they contain inactivated or highly purified microorganisms. This modulation of the immune response results in increased efficacy and allows for a reduction in the amount of injected antigen, a reduction in the number of doses of the vaccine program, and even additions of new antigens that make the vaccine more complete (Schijns & O'Hagan, 2006).

The objective of this study was to evaluate the serological response of a new inactivated pentavalent viral vaccine plus a new emulsion immunomodulator developed for use in breeder hens in Brazil, and compare it with an existing commercial vaccine. In addition, productive parameters such as mortality, weight gain, egg production, and hatching were also measured to assess the safety of the new product.

MATERIAL AND METHODS

Study 1 – Antibody response in SPF chickens

Poultry and experimental design

A group of 50 one-day-old White Leghorn *Specific Pathogen Free* (SPF) chickens were individually identified and housed in four HEPA-filtered isolators at the Zoetis Animal Facility in Paulínia (SP). Each isolator had a capacity of up to 15 animals. Prior to housing, cleaning and disinfection procedures were performed. In addition, blood samples were randomly collected from four chickens to determine the absence of maternal antibodies for all diseases investigated in this study. In this initial phase, the isolators were equipped with tubular feeders, bell drinkers, shaving litter, and lamp heating. The isolators also relied on environmental enrichment to promote animal welfare. When they reached 6 weeks of age, all chickens were transferred to 13 cages. Each cage had a maximum capacity of 4 animals. The distribution of the chickens was totally randomized, and there was at least one bird from each of the treatments in each cage. During the entire experimental period, chickens were provided with bran feed *ad libitum* and water by properly trained professionals, following lineage recommendations. The chickens received a wing washer for individual identification that remained until the end of their life, to allow for the individual serological evaluation of the animals in each treatment group.

All animals received the same live vaccines to primer the inactivated vaccine, according to the table 1:

Table 1 – Program of live vaccines used in the chickens of study 1.

Weeks	Disease	Commercial product	Route of administration
1	Metapneumovirus	Poulvac TRT	Ocular
1	Newcastle	Poulvac NDW	Ocular
1	Bronchitis	Mass I	Ocular
1	Gumboro	Bursine II	Ocular
3	Reovirus	Poulvac Reo	Wing Membrane
3	Gumboro	Poulvac Bursa F	Ocular
3	Newcastle	Poulvac NDW	Ocular
3	Bronchitis	Mass I	Ocular
3	Metapneumovirus	Poulvac TRT	Ocular
5	Reovirus	Poulvac Reo	Wing Membrane
5	Newcastle	Poulvac NDW	Ocular
5	Bronchitis	Mass I	Ocular
5	Gumboro	Poulvac Bursa F	Ocular
5	Metapneumovirus	Poulvac TRT	Ocular

This protocol was submitted to the Animal Use Ethics Committee (CEUA) of Zoetis in accordance with the rules of the National Council for Animal Experimentation (CONCEA) (Brazil, 2015). It was approved and registered under code AF 009/20 before the start of the activities.

Inactivated vaccine and vaccination process

In the 9th week of life, only the T02 poultry received a dose of the inactivated vaccine intramuscularly, with an injected volume of 0.5 mL in the left chest musculature. The poultry of the control group (T01) did not receive any viral oily vaccines. The product used in the T02 treatment was one of the pilot batches of the new pentavalent viral vaccine (Poulvac ® Maternavac® Ultra 5) recently licensed by the Ministry of Agriculture and Supply (MAPA).

Each of the vaccines used is described in the table 2:

Table 2 – Experimental design of study 1 with SPF poultry.

Treatment	Description	Batch of the vaccine	Composition of the vaccine	Number of birds
T01	Negative control	-	-	25
T02	Poulvac® Maternavac® Ultra 5	PLS 002/18	Viral inactivated suspension of Newcastle (LaSota strain), Chicken infectious bronchitis (Massachusetts type, Holland strain), Avian metapneumovirus (clone k strain subtype A), Gumboro (Lukert and 28-1 strains), Reovirus (1733 and 2408 strains) in oily emulsion containing the immunomodulator Fortilyst®	25

Sample collection

Blood samples from the 25 chickens/treatment were collected at the 9th, 11th, 13th, 15th, 17th, and 19th weeks of life of the animals. The collection was performed by means of a standard procedure (puncture of the wing vein) that was always conducted by the same person. After the blood sample was collected, serum separation, freezing and storage were performed. At the end of the study, all individually identified samples were sent to the JFLAB animal pathology laboratory (Campinas, SP) to perform the Enzyme-Linked Immunosorbent Assay (ELISA) test with a commercial kit from Biochek.

Laboratory Analysis

The ELISA test performed was indirect for antibody detection of Gumboro diseases (IBD), Avian Metapneumovirus (aMPV), Newcastle Disease (NDV), Infectious Bronchitis (IBV), and Reovirus (REO). The result was read on a spectrophotometer and the percentage of light transmission was evaluated by a reader software and converted to OD (optical density). The results of each sample/chicken were expressed as titers and categorized as positive or negative according to a cutoff value determined by the manufacturer of the ELISA kit.

Statistical Analysis

Initially, we carried out the descriptive analysis of all variables with the estimation of means, medians, standard deviations, and interquartile ranges. Then, the Shapiro-Wilk test for normal distribution was performed and the non-parametric approach was chosen for the data, since not all data presented normal distribution (Shapiro-Wilk p-value <0.05). Differences between treatments were verified with the Kruskal-Wallys test (non-parametric) followed by Dunn's test to adjust for multiple comparisons. For a better visualization of the results, boxplot charts were produced, differentiating between treatments at each moment. The difference in the percentage of positives between the groups was verified with the chi-square test and the intensity of the association was verified with the estimation of relative risk. The analyses were performed in SPSS 20.0 (IBM, 2012), and tests were considered significant when p<0.05.

Study 2 – Serological curve in commercial poultry

Poultry and experimental design

A total of 300 12-weeks-old commercial chickens of the Novogen White Light lineage were housed in 05 pens/treatment in the Veterinary Research Support Center (CAPEV) located in the municipality of Amparo - SP. The experimental design involved three treatments with 100 chickens each, being T01 – control/placebo, T02 – Poulvac® Maternavac® Ultra 5, and T03 – commercial pentavalent vaccine. 20 chickens and 2 roosters were housed in each pen (1:10). 4 chickens in each pen were also randomly assigned for blood collection, weighing and necropsy of the application site at the end of the study. Prior to housing, cleaning and disinfection procedures were performed to prevent any spread of infectious or vaccine agents to the poultry of the experiment.

In the initial phase (before 12 weeks), the poultry were raised in a commercial farm with gutter type feeders, nipple drinkers, shaving litter, and heating by campanulas. Throughout the experimental period, feed was provided following lineage recommendations. All animals received the same live vaccines to primer the inactivated vaccine according to the table 3.

This protocol was submitted to the Animal Use Ethics Committee (CEUA) of Zoetis and was in accordance with the rules of the National Council for Animal Experimentation (CONCEA) (Brazil, 2015). It was approved and registered under no. B1D94/21 before the start of the activities.

Inactivated vaccine and vaccination process

In the 14th week of life, only the T02 and T03 poultry received a dose of the inactivated vaccine intramuscularly, with an injected volume of 0.5 mL in the left chest musculature. The T01 poultry (negative

Table 3 – Program of live vaccines used in the poultry of study 2.

Weeks	Disease	Commercial product	Route of administration
1	Gumboro	BUR 706 R	Ocular
1	Newcastle	ND HB1	Ocular
1	Bronchitis	IBRAS BR	Ocular
3	Gumboro	BUR 706 R	Ocular
3	Newcastle	ND HB1	Ocular
3	Bronchitis	BIORAL H120	Ocular
6	Metpneumovirus	NEMOVAC	Spray
8	Avian Pox + Encephalomyelitis	POXIBLEN	Wing web
8	Newcastle	ND HB1	Ocular
8	Gumboro	BUR 706 R	Ocular
8	Bronchitis	IBRAS BR	OCULAR
8	E. coli	AUTOGENOUS	Intramuscular
10	Metapneumovirus	NEMOVAC	Spray
12	Reovirus	Poulvac REO	Wing web

control group) received a placebo formulation containing the same excipients as the T02 oily vaccine, except for the antigenic fractions. The product used in the T02 treatment was one of the pilot batches of the new pentavalent viral vaccine (Poulvac® Maternavac® Ultra 5) recently licensed by the Ministry of Agriculture and Supply (MAPA).

Each of the vaccines used is described in the table 4.

Sample collection

Blood samples from 4 chickens/pen were collected on the day of vaccination with the inactivated vaccines or placebo (before application) and also 13, 20, 27, 34, 41, 48, 132, 139, and 146 days after vaccination. The collection was performed by means of a standard procedure (puncture of the wing vein), and the procedure was always conducted by the same person. After the blood sample collection, serum separation, freezing, and storage were performed. At the end of the study, all identified samples were sent to the JFLAB animal pathology laboratory (Campinas, SP) to perform the ELISA test.

Laboratory Analysis

The ELISA test performed was indirect for the detection of antibodies for Gumboro, Reovirus, and Newcastle disease with the IDEXX commercial kit, and of avian Metapneumovirus, and Infectious Bronchitis with the BIOCHEK commercial kit. The result was read on a spectrophotometer and the percentage of light transmission was evaluated by a reader software and converted to OD (optical density).

Mortalities and removals

Occurring mortalities as well as poultry removed and euthanized for welfare reasons (dying poultry) were recorded daily.

Weighing

Twenty chickens from each group, as determined by the randomization plan, were individually weighed weekly.

Egg Production

The hens started to lay eggs when they were around 17-18 weeks old. From this moment onwards, all the eggs of each pen were daily harvested and counted. Moreover, from 24 weeks of age onwards, all viable eggs (without cracks, shell problems, and/or deformations) of each pen were incubated once a week for hatching evaluation.

Evaluation of the Application Site

At the end of the study, after the animals were euthanized, the application site was evaluated for

Table 4 – Experimental design of study 2 with commercial poultry

Treatment	Description	Departure of the vaccine	Composition of the vaccine	Number of poultry
T01	Negative control/placebo	NA	Oily emulsion containing FORTILYST®	100
T02	Poulvac® Maternavac® Ultra 5	PLS 001/21	Viral inactivated suspension of Newcastle (LaSota strain), Chicken infectious bronchitis (Massachusetts type, Holland strain), Avian metapneumovirus (clone k strain subtype A), Gumboro (Lukert and 28-1 strains), Reovirus (1733 and 2408 strains) in oily emulsion containing the immunomodulator FORTILYST®	100
T03	Commercial pentavalent vaccine (conventional)	012/20	Vaccine against Newcastle disease (La Sota), Reovirus, Chicken infectious bronchitis (H-120) and Gumboro (GBV-8 and 1084-E), cultured in embryonated eggs of SPF chickens, and against swollen head syndrome (SHS 119/95-BR and TRTV-BR), cultured in cells of SPF chicken embryos, inactivated and micro emulsified.	100

the presence of possible macroscopic lesions. For this evaluation, the same poultry randomized for the weighing and blood collection procedures (20 birds/group) were necropsied.

Statistical Analysis

The mortality percentage was calculated for each treatment, with the number of poultry allocated in each pen associated with the respective treatments being used as the denominator for the calculations. Mortality results were analyzed with a mixed generalized linear model for binomial distribution with logit link. The model included the fixed effect of the treatment, as well as the random effects of the block and its interaction with the treatment. Mortality was analyzed considering the total period of the study.

Body weight, egg production, percent of egg hatching and serological results were analyzed by a general linear mixed model for repeated measures. For this analysis, the fixed effects of treatment group, experimental moment and their interactions were considered. Regarding results collected at the animal level (body weight and serological results), the random effects of the block, the interaction between block and treatment, the animal within the block and treatment, the interactions between block, treatment and experimental moment, and the error were considered. For results collected at the pen level (egg production and egg hatching percentage), the random effects of block, the interaction of block and treatment, and the error were considered. Serological results were log transformed prior to analysis, and the egg hatching percentage was arcsine square root transformed prior to analysis. The analysis considered a significance level of 5% ($p \leq 0.05$). Least squares means, standard errors, and 95% confidence limits were re-transformed (BT LSmeans) as appropriate for each experimental time point. Minimum and maximum values were calculated for each experimental moment. When there was a significant effect of the treatment group or treatment group by experimental moment ($P \leq 0.05$), experimental groups T01, T02 and T03 were compared at each moment using contrasts.

Weight gain and total egg production considering the total study period were analyzed with a general linear mixed model for binomial distribution with logit link. The model included the fixed effect of the treatment. Regarding weight gain, the random effects included block, its interaction with the treatment, and error. For total egg production, the random effects included block and error.

RESULTS

Study 1 – Serological curve in SPF poultry

Figure 1 shows the boxplot graphs of the quantification of serology in SPF poultry for IBD, REO, NDV, IBV, and aMPV in each study treatment 1 on days 14, 28, 42, 56, 70 and 84 after vaccination (ELISA – IDEXX kit).

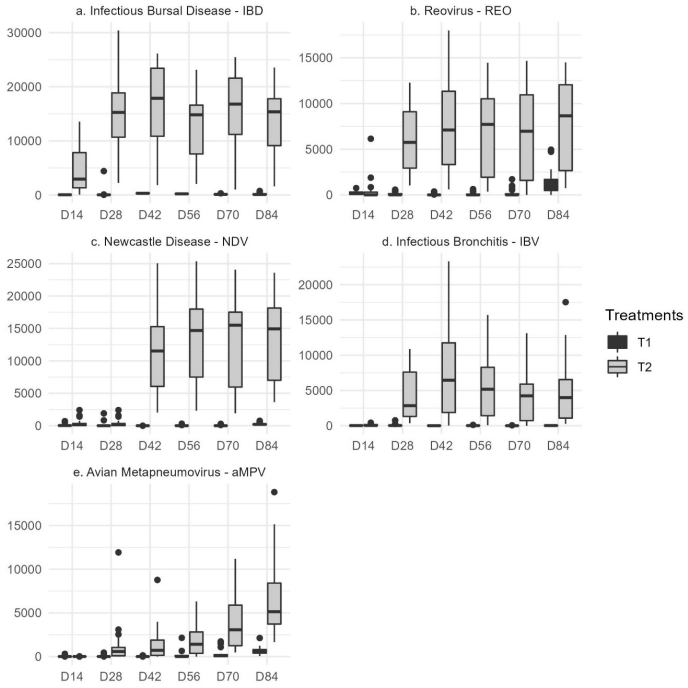

Figure 1 – Boxplot graphs of the serology quantification in SPF poultry for IBD, REO, NDV, IBV and aMPV in each study treatment on days 14, 28, 42, 56, 70, and 84 after vaccination (ELISA – IDEXX).

Study 2 – Serological curve in commercial poultry

Figure 2 shows the graphs of serology quantification in commercial poultry vaccinated with Poulvac® Maternavac® Ultra 5 compared with a conventional pentavalent vaccine for the IBD, REO, NDV, IBV and aMPV fractions on days 0, 13, 20, 27, 34, 41, 48, 132, 139 and 146 after vaccination (ELISA – IDEXX and BIOCHEK).

DISCUSSION

The development of high performance and safe veterinary vaccines requires the improvement of formulations and often the use of components that can modulate the immune response to induce better performance when compared to conventional adjuvants and formulations (Manuja *et al.*, 2013). Adjuvants and immunomodulators potentiate the presentation of immunogens to cells of the immune

Study 2 – Mortality and production data

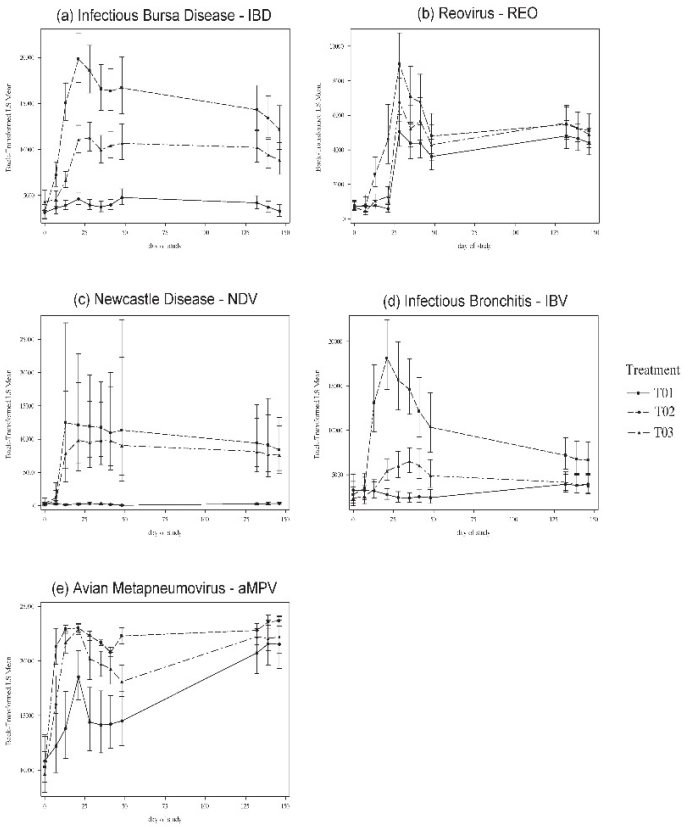

Figure 2 – Graphs of serology quantification in commercial poultry vaccinated with Poulvac® Maternavac Ultra 5 compared with a conventional pentavalent vaccine for the IBD, REO, NDV, IBV and aMPV fractions on days 0, 13, 20, 27, 34, 41, 48, 132, 139, and 146 after vaccination (ELISA – IDEXX and BIOCHEK).

system in order to initiate or enhance the response through affinity with the Toll-like receptor (Vollmer & Krieg, 2009; Fehér, 2019). Immunomodulators are also able to redirect the immune response, making it more potent and even safer, as they can reduce the deleterious effects of some components (Cserep, 2009; Scheiermann & Klinman, 2014). The formulation of this new pentavalent inactivated vaccine includes Fortilyst®, which has an immunomodulatory action that improves the response to viral antigens (IBD, REO, NDV, IBV and aMPV).

Studies have been conducted to measure the level of transfer of maternal antibodies and it is well established that the rate of transfer varies from disease to disease. In Gumboro disease, for example, there is a transfer of up to 70% of the mother's antibody level to its progeny (Gharaibeh *et al.*, 2008). This maternal immunity is an important tool for the early protection of the progeny since there invariably are viral challenges during the first days of chicks' lives. The greater the amount of antibodies produced by the inactivated vaccine in the breeder, the greater the level of antibodies transferred to the progeny tends to be (Ahmad & Akhter, 2003).

The genetic variability of the field challenge is also an important aspect in the protection of maternal immunity, and in Brazil, in addition to classical viruses, Gumboro variant viruses have also been also identified (Muniz *et al.,* 2018; de Fraga *et al.,* 2019). Thus, in addition to the amount of antibodies, their quality and diversity are also important factors in the protection of the progeny.

Inactivated vaccines for Gumboro disease aim to stimulate the production of high levels of antibodies in breeders. These should be used in poultry that have already been sensitized by live vaccines or even by the field virus, which can be evaluated serologically (Dey *et al.*, 2019). Considering that the use of inactivated vaccines generally occurs from the 12th week of life onwards, it is known that within a primary vaccination program with live vaccines, the evaluation of the serological curve is an important parameter of vaccine efficacy. In addition, the correlation between poultry protection and antibody levels has already been described in the literature (Maas *et al.,* 2001; Bolis *et al.,* 2003).

In the construction of a vaccination program for breeding animals, it is extremely important to think not only about the protection of the breeders themselves, but also about the protection of the progeny. This is particularly true when talking about Gumboro disease, as chicks in their first weeks of life are dependent on maternal antibodies for effective protection against it (Van Den Berg *et al.,* 2000). The booster effect of vaccination with Poulvac® Maternavac® Ultra 5 for the IBD fraction was measured and is shown in figure 1a. In the comparison between the two vaccines, after 13 days of vaccination, it was possible to observe higher average titers ($p \leq 0.05$) in the T02 group when compared to T01 and T03 groups (Figure 2a). The correlation of higher antibody titers in hens leading to higher antibody titers in the progeny is well established and, consequently, these chicks will be better protected.

Malabsorption syndrome and viral arthritis, both caused by the Reovirus, are challenges observed in Brazilian poultry farming that can cause great losses to producers and companies when neglected (Souza *et al.,* 2018). During the first weeks of life, chicks are highly dependent on maternal antibodies to control Reovirus and Gumboro health challenges in the field (Edison *et al.*, 1979; Van Der Heide & Page, 1980; Jones & Georgiou, 1984; Jones & Nwajei, 1985; Martins & Resende, 2009). Therefore, immunoglobulins

transferred from breeders can avoid significant losses (particularly in chicks' locomotor and digestive systems) if they present a high homology with field challenges (Rau *et al.*, 1980; Kant *et al.*, 2003; Liu *et al.*, 2003; Day & Pantin-Jackwood, 2007; Jones, 2008; Sellers, 2017). In Figure 1b of study 1, it is possible to observe the booster effect related to the REO fraction from 28 days after vaccination onwards. In study 2, the mean titers were similar ($p \leq 0.05$) for the T02 and T03 groups and differed significantly from T01 (Fig 2b). These findings show that the Poulvac ® Maternavac ® Ultra 5 vaccine generates high titers in poultry and, consequently, presents greater potential for progeny production.

In Brazil, Newcastle disease was included in a National Contingency Plan in 2009, which included the determination of the sacrifice of affected poultry to keep the national poultry flock free from it (Brazil, 2009). It is also considered a notifiable disease and has an impact on the international marketing of poultry products (Brazil, 1994).

The evaluation of the efficacy of inactivated vaccines against Newcastle disease is based on the determination of circulating antibodies, and the induction of seroconversion in long-lived poultry has a fundamental role in the protection of the breeder, while also contributing to the progeny in their first weeks of life through passive immunity (Dimitrov *et al.*, 2017). In Figure 2c of study 2, it is possible to observe that the average titers for NDV were similar ($p \leq 0.05$) for the T02 and T03 groups throughout the evaluation period. The result obtained 13 days after vaccination shows the booster effect. On the other hand, in Figure 1c of study 1 with SPF poultry, the induction of seroconversion of the group vaccinated with Poulvac® Maternavac® Ultra 5 was evident 42 days after vaccination, with the maintenance of the antibody plateau being observed after this age. This difference in the speed of seroconversion for NDV comparing SPF and commercial poultry may be related to the response of the two genetic strains used in the studies (Zou *et al.,* 2020).

The etiological agent that causes avian infectious bronchitis belonging to the family *Coronaviridae*, genus *Gammacoronavirus* (group 3), subgenus *Igacovirus*, is characterized by having a positive and non-segmented sense, single RNA-stranded genome. Chicken infectious bronchitis is a highly contagious acute respiratory disease, which affects poultry of the *Gallus* species and has great economic importance. The most common symptoms include nasal discharge, lacrimation, cough, sneezing, rales, and lethargy. In addition to respiratory symptoms, the avian infectious bronchitis virus can cause reproductive changes and nephritis (Ignatovic & Sapatos, 2000; Cavanagh & Gelb, 2008).

Cavanagh (2003) described that the efficacy of an inactivated vaccine for protection against IBV infections depends heavily on a suitable program with primary vaccinations with live vaccines. This characteristic could be observed in the study conducted by Santos *et al.* (2019), where the activation of immune memory after the use of a live attenuated vaccine (IBV-Mass) followed by the administration of an inactivated vaccine (inat IBV-BR1) conferred protection against the challenge with the IBV-BR1 strain. This protection against the challenge was only complete due to the use of the inactivated vaccine, since the live attenuated vaccine (IBV-Mass) would be unable to guarantee complete protection by itself.

In study 2, when comparing the two inactivated vaccines in commercial poultry, it was possible to observe higher averages ($p \leq 0.05$) for the fraction of IBV from day 13 post-vaccination onwards in the T02 group when compared to the T01 and T03 groups by the ELISA method (Figure 2d). Moreover, it was possible to observe the long-lasting effect of the vaccine response during the evaluation period (146 days post-vaccination), in which titers greater than 833 (reactivity threshold or kit cut-off) were observed. On the other hand, in study 1 with SPF poultry, we observed the induction of seroconversion from 28 days after vaccination onwards (Figure 1d).

Vaccination programs with live vaccines followed by inactivated vaccines are consolidated strategies and are widely used in industrial poultry production (Bhuiyan *et al.,* 2021) for various poultry diseases, including IBV. The high level of antibodies generated by vaccination with injectable inactivated products preceded by immunization with a live vaccine plays a decisive role in the protection of long-lived poultry, as this immunity is very effective to control systemic IBV infection, even in cases of nephropathogenic pathotypes (Landman *et al.,* 2002; Santos *et al.*, 2019).

It is known and reported in the literature and in field observations that aMPV is a weak inducer of seroconversion, but when there is high infection pressure, serology is the most practical method to perform risk monitoring and analysis (Park *et al.*, 2011). Furthermore, the OIE guide (2018) recommends that the evaluation of the efficacy of inactivated vaccines that have aMPV in their composition should be carried out by assessing the serological response of animals,

given the great difficulty of reproducing challenge conditions for the evaluation of variables related to aMPV infection (Turpin *et al.*, 2002; Gough & Jones, 2008). Live vaccines, without the presence of challenge, do not result in high circulating antibody titers, and seroconversion is quite slow when compared to other diseases. In addition, aMPV is difficult to isolate, as it stays present for a short period of time, being detected only at the beginning of clinical signs in infected poultry (Cook & Cavanagh, 2002).

The greatest differences between the designs of the serological curves of the two studies were observed in the aMPV fraction. In study 1 (Figure 1e) with SPF poultry, a slow seroconversion was observed, consolidating in the last week, that is, 84 days after vaccination. On the other hand, in study 2 (Figure 2e) the result obtained as soon as 7 days after vaccination shows the booster effect of the Poulvac® Maternavac® Ultra 5 vaccine. Moreover, it was possible to verify the long-lasting effect of the vaccine response, with significantly high titers until 146 days after vaccination. The curve of study number 2 indicates that in addition to the effect of the vaccine, there may also have been a field challenge, since the poultry of the experiment were commercial and were recreated in a field environment with the possibility of being exposed to a field virus.

Studies published in Brazil have reported seroconversion in unvaccinated poultry, proving that the agent is present in industrial farms (Peres *et al.,* 2006) and in free-range poultry (Sales *et al.,* 2010). In any case, this serological profile observed in study 2 indicates the excellent ability of vaccines to produce protection, since the appearance of clinical signs of the disease was not observed at any time during the study. The intense seroconversion demonstrated that there was exposure to the agent, but the poultry were protected by the vaccination program with live and inactivated vaccines and the serological response was enhanced in T02 by the immunostimulant (Bode *et al.*, 2011).

The induction of early – and long – term antibodies and their duration in poultry vaccinated with the new inactivated pentavalent vaccine was observed for each of the antigenic fractions present in the formulation of the Poulvac® Maternavac® Ultra 5, considering the serological responses of the animals included in both studies. It was possible to establish the serological response curve of the neutralizing antibodies that are the basis of the evidence of efficacy of this type of product. Also, it is important to note that before the administration of the inactivated vaccine, the poultry in groups T02 and T03 were submitted to the administration of an extensive program of primary vaccines with live vaccines, which aimed to mimic the real field conditions in which the vaccine will be used when it is available on the market (Brazil, 2006).

The use of Fortilyst® (immunomodulator) was shown to be effective to induce the appropriate antigenic immune response (Figures 1 and 2). In addition, it is presented as an alternative for the preparation of multivalent products with new antigenic fractions in the formulation of vaccines (Shirota & Klinman, 2014; Shirota *et al.*, 2015). Moreover, study 2 demonstrated that the administration of a single dose of the Poulvac ® Maternavac ® Ultra 5 vaccine in breeder hens did not cause clinical, behavioral or increased animal mortality changes (Table 5). In the macroscopic evaluation, no signs of tissue necrosis, edema, changes in muscle color, presence of nodules or hemorrhage were observed. In the three groups, poultry with neovascularization near the application site were observed, which is expected for inactivated vaccines in general.

Table 5 – Mortality analysis – means (BT LS means) and confidence intervals per treatment.

Group	Treatment	Box/ Group	Average (BT LS Means)[1] (%)	Standard Error	Lower confidence limit 95%	Upper confidence limit 95%	Minimum	Maximum
T01	Control/Placebo	5	0.20	0.41	0.35	2.21	0.0	5.0
T02	Poulvac® Maternavac® Ultra 5	5	0.20	0.41	0.35	2.21	0.0	5.0
T03	Conventional commercial vaccine	5	0.20	0.41	0.35	2.21	0.0	5.0

[1]Treatments were not significantly different (p=1.00)

Weight gain throughout the study was similar between the groups, with no negative impact on the weight of vaccinated poultry being verified, especially when compared with the negative control group (Table 6 and Figure 3). Body weights of the Poulvac® Maternavac® Ultra 5 group (T02) were significantly higher than those of negative control group (T01) from day 77 until the end of the study, although there were no significant differences in average daily weight gain. The production of eggs by poultry vaccinated with the tested vaccine was similar to that of the control groups (Negative – T01 and Positive – T03), considering the total average of eggs had no significant differences

Table 6 – Analysis of average daily weight gain (g/day) for the three experimental groups (T01, T02 and T03) in the total study period (D0 to D+151)

Group	Treatment	Animals/ group	Average (BT LS Means)[1]	Standard Error	Lower confidence limit 95%	Upper confidence limit 95%	Minimum	Maximum
T01	Control/Placebo	20	3.30	0.137	2.98	3.61	1.80	4.39
T02	Poulvac® Maternavac® Ultra 5	20	3.83	0.137	3.52	4.15	2,10	4.81
T03	Conventional commercial vaccine	20	3.61	0.137	3.29	3.93	2.91	4.86

[1]Treatments were not significantly different (p=0.07)

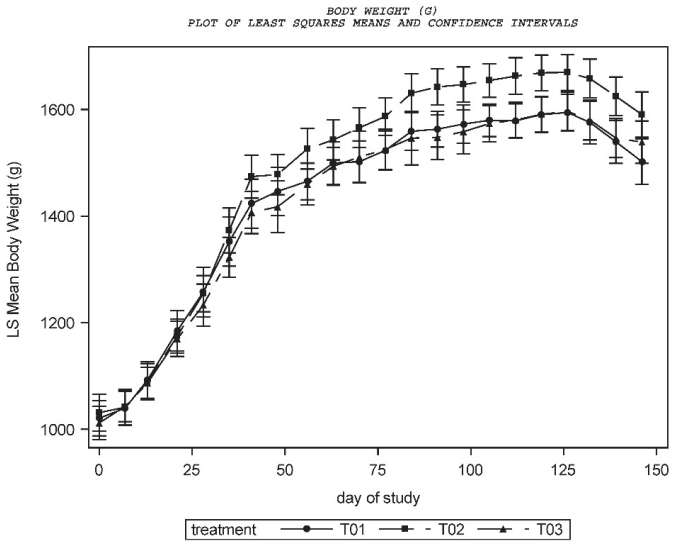

Figure 3 – Mean (BT LSmeans) and standard error of body weight obtained from poultry in study 2 between days D0 and D+151.

between groups (Table 7). The percentage of egg hatching was also similar between poultry vaccinated with Poulvac® Maternavac® Ultra 5 (T02) and the control groups (Negative – T01 and Positive – T03) (Figure 4), with no significant differences between groups. All these data prove the safety of the vaccine.

The oligonucleotide (ODN) called CpG has gained considerable prominence among the various types of existing immunomodulators. CpG oligonucleotides are small single-stranded DNA molecules of defined sequence and size containing nucleotide bases of Cytosine (C) followed by Guanine (G) (Krieg, 2002; Krieg, 2012). This nucleotide sequence is widely found in the genetic material of viruses and bacteria, but rarely found in vertebrate genomes. One difference between the sequence of CpG oligonucleotides found in vertebrates and the CpG ODNs found in viruses and bacteria is that in vertebrates this sequence is methylated. The synthetic CpG ODN molecule mimics sequences naturally found in viruses and bacteria. Just as the DNA of a pathogen, synthetic CpG ODN has been shown to stimulate innate protection as well as adaptive immune responses, therefore serving as an immunomodulator (Klinman *et al.*, 2009; Salem & Weiner, 2009; Bode *et al.*, 2011).

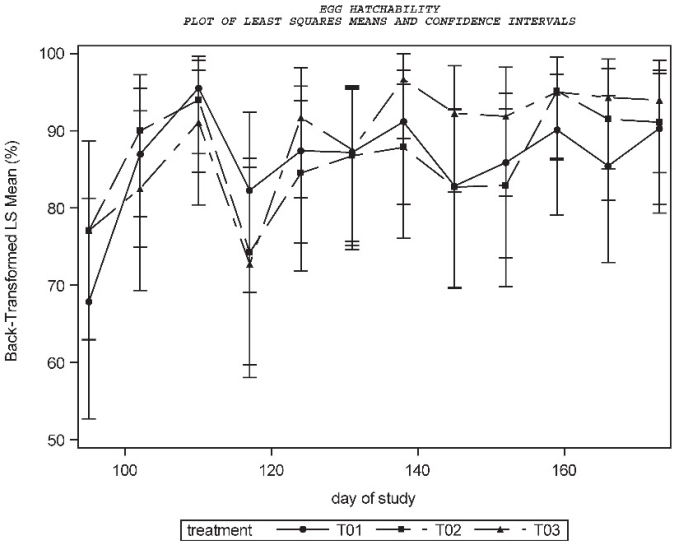

Figure 4 – Percentage of hatching (BT LSmeans) and standard error obtained for groups T01, T2, and T03 of study 2.

There are several publications describing and evaluating 3 different classes of CpGs, referred to as class A, B and C CpGs. Most studies in the literature have used class B CpGs as adjuvants in vaccines (Vollmer *et al.* 2004), but more recently, a new, different class has been developed. This new compound has undergone modifications to improve its immunostimulatory activity and is called 'E-modified P-class CpG' or 'EP-CpG' (Samulowitz *et al*, 2010).

Table 7 – Means (BT LSmeans) of egg production per box in the total period evaluated (D+27 to D+149) for experimental groups T01, T02 and T03.

Group	Treatment	Box/ group	Average eggs/box (BT LS means)[1]	Standard Error	Lower confidence limit 95%	Upper confidence limit 95%	Minimum	Maximum
T01	Placebo	5	2062.0	12.84	2032.5	2091.8	2011	2104
T02	Poulvac® Maternavac® Ultra 5	5	2082.3	12.97	2052.6	2112.4	2053	2105
T03	Conventional commercial vaccine	5	2103.0	13,09	2073.0	2133.5	2065	2131

[1]Treatments were not significantly different (p=0.14)

Modified CpG ODNs associated with viral antigens in an oily emulsion enable better antigen presentation by stimulating Th1 cells via TLR-9, thereby improving the immune response with superior antibody production (McCluskie & Krieg, 2006; Jurk & Vollmer, 2007). The synthetic CpG ODN interacts with Toll-Like Receptor 9 receptors present in the innate immune system cells of poultry and act as agonists. The TLR-9 agonist activity of synthetic CpG ODN was shown to stimulate both innate protection and adaptive immune response (Donhauser *et al.*, 2010; Meng *et al.*, 2016).

Therefore, based on experimental studies in both SPF and commercial poultry, the Poulvac® Maternavac® Ultra 5 vaccine was shown to be safe and have proven efficacy in the protection against diseases caused by the IBD, REO, NDV, IBV and aMPV viruses through the induction of seroconversion when used in revaccination protocols in the target species, as recommended in the product package insert. Multivalent protection was demonstrated through seroconversion curves and was enhanced by Fortilyst® (immunomodulator). The results showed that the addition of the immunomodulator was essential to induce adequate levels of seroconversion and contribute to the enhancement of the immune response, in line with previous literature reports on the immunomodulatory effect of this molecule.

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