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# Implementation of a "DIVA" Concept with specific Elisa Kits; When Subunit H5 Avian Influenza Vaccine is used

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***Abstract*—**The main objective of this study was to demonstrate that differentiation of infected and vaccinated animals (DIVA) strategy using different ELISA tests is possible when a subunit vaccine (Haemagglutinin protein) is used to prevent Avian influenza. Special emphasis was placed on the differentiation in the serological response to different components of the AIV (Nucleoprotein, Neuraminidase, Haemagglutinin, Nucleocapsid) between chickens that were vaccinated with a whole virus kill vaccine and recombinant vaccine. Furthermore, the potential use of this DIVA strategy using ELISA assays to detect Neuraminidase 1 (N1) was analyzed as strategy in countries where the field virus is H5N1 and the vaccine used is formulated with H5N2.

Detection of AIV's antibodies to any component in serum was negative for all animals on the study days 0-13. At study day 14 the titers of antibodies against Nucleoprotein (NP) and Nucleocapsid (NC) rose in the experimental groups vaccinated with Volvac® AI KV and were negatives during all the trial in the experimental groups vaccinated with a subunit H5; significant statistically differences were observed between these groups ($p < 0.05$). The seroconversion either Haemagglutinin or Neuraminidase was evident after 21 days post-vaccination in the experimental groups vaccinated with the respective viral fraction.

Regarding the main aim of this study and according with the results that were obtained, use a combination of different ELISA test as a DIVA strategy is feasible when the vaccination is carry out with a subunit H5 vaccine. Also is possible to use the ELISA kit to detect Neuraminidase (either N1 or N2) as a DIVA concept in countries where H5N1 is present and the vaccination programs are done with H5N2 vaccine.

***Keywords*—**Avian Influenza Virus; "DIVA concept"; ELISA assay; subunit H5 vaccine.

## I. Introduction

THE vaccination is being considered more commonly today as an alternative control to stamping out for Avian Influenza because it is viewed as a more cost effective control strategy [2], [3]. However, vaccination can affect serologic surveillance and have negative impacts on international trade. For both trade and surveillance purposes, it is important not only to differentiate naturally infected and vaccinated chickens, but also to identify vaccinated chickens that become infected with Avian Influenza Virus (AIV) [1], [4]. Historically, one limitation of the killed vaccines is that vaccinated chickens cannot be differentiated serologically from naturally infected chickens using the commonly available diagnostic tests.

Therefore, surveillance for Avian Influenza becomes much more difficult and often results in trade restrictions because of the inability to Differentiate Infected from Vaccinated Animals (DIVA) [1]. Some different DIVA strategies have been proposed to overcome these barriers to vaccination:

A) *Sentinel chickens*: use of unvaccinated sentinels in vaccinated flocks to determine if they have been exposed to AIV (this is the most common strategy). Routinely the sentinels are tested by standard serologic test, and direct virus detection methods. The main disadvantage is related with the management of the sentinel birds.

B) *Subunit vaccines*: Because antibodies to the Haemagglutinin and neuraminidase proteins provide the primary protection, it is possible to protect birds by having only these proteins in a vaccine. Many different types of subunit vaccine, including virus vectored vaccines and vaccines using protein expressed in different culture system.
Vaccinated birds will not develop antibodies to the internal proteins, providing a clear distinction between infected (has antibodies to matrix or nucleoprotein proteins) and vaccinated bird (has antibodies to Haemagglutinin only). The biggest drawback; particularly for the live viral vectors is regulatory, for to be genetically modified organisms.

C) *Heterologous neuraminidase strategy*: The AIV has 10 different proteins, including Hemagglutinin (HA) and neuraminidase (NA). Therefore, standard killed vaccine with heterologous NA subtype can be use effectively, and this allows the possibility of a DIVA strategy based on serologic test target to the NA protein. For example, if the circulating field strain of virus is H7N2, chickens can be vaccinated with H7N3. Serologic monitoring to the N3 protein can confirm that the flock has been vaccinated; a serologic monitoring to the N2 can to demonstrate infection with field virus.

D) *NS1 detection*: Has been described than the NS1 protein is produced in large amounts in infected cells, but it is not present in killed virus vaccine; theoretically the animals vaccinated should not have an antibody response to the NS1

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protein. This DIVA approach was demonstrated with equine influenza virus. However, commercial avian influenza vaccines are made with allantoic fluid only partially purified, therefore, they contain small amounts of NS1 protein in the vaccine and the chickens will develop some antibodies. The difference in antibody levels can be used to development a reliable DIVA diagnostic test. The primary advantage is the ability to work with any killed vaccine using a single diagnostic test. The major disadvantage is the potential contamination with NS1 protein in the manufacture of the vaccine [4].

In this study, ninety SPF chickens received either a subunit H5 vaccine or conventional whole killed virus vaccine by subcutaneous injection; there was daily monitoring for the evaluation of the general health parameters. Three different Enzyme-linked immunosorbent assay [ELISA] tests; able to detect antibodies against either Hemagglutinin H5 [Orgenics, Israel and IDVet, France] or Nucleoprotein [ImmTech, USA] or Neuraminidase [IDVet, France], were used as a DIVA concept. The main objective of this study was to demonstrate that the Differentiation of Infected and Vaccinated Animals (DIVA) strategy using different ELISA tests is possible when a subunit H5 vaccine is used to prevent Avian Influenza.

## II. Materials and Methods

This study was conducted in chickens SPF at age of 1 day, under field conditions. 2 different vaccines against AIV were used; these vaccines claim protection against Avian Influenza Virus and the immune response was monitored with 3 commercial ELISA kits in order to use them as a DIVA concept. The experimental Unit was every experimental group of chickens, because the analysis (Analysis of variance test - ANOVA) of the average antibodies titers was enough to obtain the information.

There were 3 treatment groups with around 30 chickens in each group; 2 of these groups (T1 & T2) were vaccinated either subunit H5 vaccine or whole virus kill vaccine (Volvac® AI KV, Boehringer Ingelheim Vetmedica S.A. de C.V.) and the negative control group was vaccinated with a placebo. Groups T1 & T2 were vaccinated by administration of the commercial dose (0.5 ml) subcutaneous via, at age of 10 days. The chickens were recruited in different cage, according with the experimental design, and each group was identifying by different color.

There were 5 samplings (at 0, 14, 21, 28 and 35 days pos vaccination); the blood samples were taken with sterile syringes and were keep at 4 - 8 °C, after that the sampling was finished the samples were sent (following the cold chain 4 – 8 °C) to the laboratory site (Boehringer Ingelheim Vetmedica S.A. de C.V., Mexico) for their analysis with the ELISA kits following the directions from the supplier. The results were analyzed by ANOVA test with SAS software release 8.2 (2001) (SAS, Cary, North Carolina: SAS Institute Inc).

## III. Results

The antibody titers of the placebo and subunit H5 vaccine groups showed rise at 28 Post-vaccination; however, always stay negative. The experimental group vaccinated with Volvac ® AI KV showed seroconvertion at 14 days post-vaccination, there is a significant statistical difference ($p < 0.5$) between the groups vaccinated with Volvac ® AI KV (the whole virus) and they which did not vaccinated with Volvac ® AI KV. That means is possible to differentiate between chickens that have been exposed to the Avian Influenza virus from the chickens that have not been in contact with it. Table I presents the antibody titers of Nucleoprotein- positive chickens over the course of the study.

TABLE I
Mean antibody titers against Nucleoprotein (NP) of the different treatment groups, using ImmTech ELISA kit.

| PV DAYS/ GROUP | Placebo | Subunit H5 | Volvac AI |
|---|---|---|---|
| | 10 days old | | |
| 0 | -176.131 | -138.257 | -124.666 |
| 14 | -187.752 | -151.25 | **43.75765** |
| 21 | ND | 2.045159 | **85.53068** |
| 28 | 4.847657 | 3.569721 | **95.7654** |
| 35 | 1.845986 | 8.087971 | **98.92088** |

Note: PV= Post-vaccination days; ND= Do not done. The grayish box means that the titers are considered positives.

The antibody titers of the placebo were steady, always stay negative. The group of subunit H5 alone showed increase from 14 days post-vaccination, but until 28 days post-vaccination the mean of the titers crossed the threshold to be considered positive. The experimental group vaccinated with Volvac ® AI KV showed increase from 14 days post-vaccination, but until 35 days post-vaccination the mean of the titers crossed the threshold to be considered positive. There is a significant statistical difference ($p < 0.001$) between the groups vaccinated with Volvac ® AI KV (the whole virus) and they which did not vaccinated with Volvac ® AI KV. Table II presents the antibody titers of Haemagglutinin H5-positive chickens (using the ELISA kit from Orgenics) over the course of the study.

The antibody titers of the placebo group was steady, always stay negative. The experimental group of subunit H5 alone showed increase from 14 days post-vaccination, but until 28 days post-vaccination the mean of the titers crossed the threshold to be considered positive The experimental group vaccinated with Volvac ® AI KV showed increase from 14 days post-vaccination, but until 21 days post-vaccination the mean of the titers crossed the threshold to be considered positive, there is a significant statistical difference ($p < 0.001$) between the groups vaccinated with Volvac ® AI KV (the whole virus) and they which did not vaccinated with Volvac ® AI KV. Table III presents the antibody titers of Haemagglutinin H5- positive chickens (using the ELISA kit from ID Vet) over the course of the study.

TABLE II
MEAN ANTIBODY TITERS AGAINST HAEMAGGLUTININ 5 (H5) OF THE DIFFERENT TREATMENT GROUPS, USING ORGENICS ELISA KIT.

| PV DAYS/ GROUP | Placebo | Subunit H5 | Volvac AI |
|---|---|---|---|
| | 10 days old | | |
| 0 | 2.584993 | -7.45066 | -2.1972 |
| 14 | 22.10396 | 27.25854 | 21.79553 |
| 21 | -4.84289 | 40.27106 | 14.91255 |
| 28 | 22.45836 | **58.26539** | 36.56794 |
| 35 | -2.68345 | **74.50772** | **54.96397** |

Note: PV= Post-vaccination days; ND= Do not done. The grayish box means that the titers are considered positives.

TABLE III
MEAN ANTIBODY TITERS AGAINST HAEMAGGLUTININ 5 (H5) OF THE DIFFERENT TREATMENT GROUPS, USING FLUAC ELISA KIT.

| PV DAYS/ GROUP | Placebo | Subunit H5 | Volvac AI |
|---|---|---|---|
| | 10 days old | | |
| 0 | 86.81278 | 91.01326 | 99.03246 |
| 14 | 94.81375 | 76.01505 | 61.00387 |
| 21 | 94.23911 | 44.25301 | **21.01202** |
| 28 | 89.08374 | **27.64388** | **8.80133** |
| 35 | 98.3324 | **22.06718** | **6.638584** |

Note: PV= Post-vaccination days; ND= Do not done. The grayish box means that the titers are considered positives.

Figure 1 presents the serological behavior of the placebo group over the course of the study with the 3 different ELISA kits that were used in the analysis. In both viral fractions analyzed (NP and H5) the titers were steady and the seroconvertion was negative from 0 to 35 days post-vaccination, as was expected. Even though in the NP fraction there is a rise after 14 days post-vaccination the titer is consider negative according with the threshold.

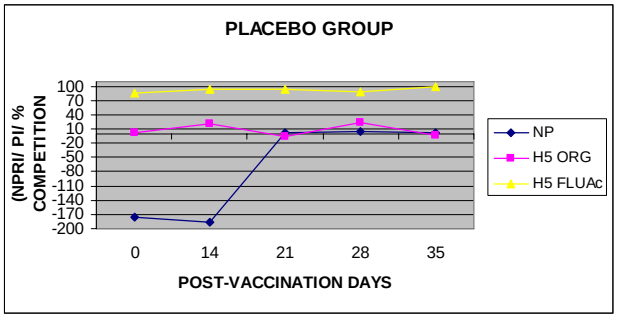


Figure1. Mean antibody titers against Haemagglutinin 5 (H5) and Nucleoprotein (NP) of the placebo group on study days 0, 14, 21, 28 and 35.

Note: Thresholds: NP >30; H5 Orgenics >50 and H5 FluAc (IDVet) <35.

Figure 2 presents the serological behavior of the subunit H5 (vaccinated at 10 days of age) group over the course of the study with the 3 different ELISA kits that were used in the analysis. The titers against NP were steady and the seroconvertion was negative from 0 to 35 days post-vaccination, as was expected, even though there is a rise after 14 days post-vaccination the titer is consider negative according with the threshold. The results from the ELISA kit H5 (Orgenics) show a constant increasing from 14 to 35 days post vaccination (at 28 days is when the titers crossed the threshold); the same behavior is observed with the results from ELISA kit (FLUAc), but the difference regarding the red out with this kit is that minor values means highest titers.

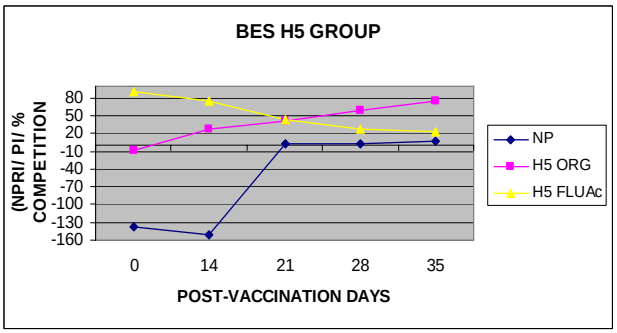


Figure 2. Mean antibody titers against Haemagglutinin 5 (H5) and Nucleoprotein (NP) of the Baculo H5 group on study days 0, 14, 21, 28 and 35.

Note: Thresholds: NP >30; H5 Orgenics >50 and H5 FluAc (IDVet) <35.

Figure 3 presents the serological behavior of the Volvac ® AI KV (vaccinated at 10 days of age) group over the course of the study with the 3 different ELISA kits that were used in the analysis. The titers against NP showed rise in seroconvertion from 14 to 35 days post-vaccination, as was expected, the titers were positives at 21 days post-vaccination. The results from the ELISA kit H5 (Orgenics) show a slightly increasing from 14 to 35 days post vaccination (at 35 days is when the titers crossed the threshold); but with the ELISA kit (FLUAc) the rise were clear from 14 days pos-vaccination and the threshold was crossed at 21 days, two weeks before that with the Orgenics kit.

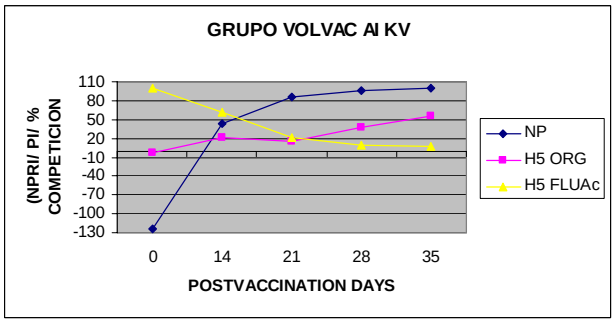


Figure3. Mean antibody titers against Haemagglutinin 5 (H5) and Nucleoprotein (NP) of the Volvac AI KV group on study days 0, 14, 21, 28 and 35.

Note: Thresholds: NP >30; H5 Orgenics >50 and H5 FluAc

(IDVet) <35.

## IV. Discussion and Conclusions

The main objective of this study was to develop and implement a DIVA strategy, based on the use of ELISA test, to the Subunit H5 prototype vaccine. Further objective was to analyze the feasibility of use a couple of ELISA kits as DIVA strategy when the Volvac ® AI KV vaccine is used in places where the Avian influenza virus present is not subtype H5 N2. Special emphasis in this study was placed on the experimental groups vaccinated at 10 days of age because is the target.

In total 90 SPF chickens were included in this study. Vaccinated and placebo- treated chickens were randomize distributed. The experimental groups were vaccinated according with the study design either the vaccine or placebo. All the treatments were given via the subcutaneous route in the 3rd back part of the neck.

Over the course of the study, the percentage of mortality was into the normal parameters; regarding the serological response, the fact that over the course of the trial there was not seroconvertion to any fraction (NP, H5, NC, N1 and N2) analyzed of AIV in the placebo groups, and the experimental groups that were vaccinated with subunit a vaccine did not show seroconvertion to a fragment that is not present in the product is clear that there is not cross reaction between the antibodies against the different fractions. On the other hand, when the chickens face a field challenge (in this study the experimental group vaccinated with the whole virus = Volvac ® AI KV) the detection of antibodies against NP are detectable at 14 days post-vaccination, that according with the results from this study this immune response is faster that the response against Haemagglutinin H5 (seroconvertion at 28 days post-vaccination).

In the case of subunit H5 group, both ELISA kits that detect Haemagglutinin H5 are capable to detect seroconvertion at 28 days post-vaccination.

In conclusion based on the results from this study is possible to differentiate vaccinated from infected (vaccinated with the whole virus) chickens when Baculo H5 is used as vaccination program; this DIVA strategy is a feasible option.

About the use of DIVA strategy using ELISA kits to detect either N1 or N2 fractions, the results show that is feasible in places where the field virus has neuraminidase 1, but not neuraminidase 2; and the chickens are vaccinated with Volvac ® AI KV (based on H5N2 AIV), this difference in seroconvertion can be observed after 14 days post-vaccination.

Finally, one interesting finding is that there is a difference in the results (days to observe seroconvertion) depending what kit is used (Orgenics is produced with H5N1 AIV and IDVet with H5N2 AIV); this is a similar behavior observed in the HI assays when depending of the homology of the antigen used is the sensibility of the test, in summary the monitoring of antibodies induce by subunit H5 is better when the Orgenics' ELISA kit is used and the monitoring of antibodies induce by Volvac ® AI KV is better when the IDVet ELISA kit is used.

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