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Assessment of the immune response in broilers and pullets using two ELISA kits after in ovo or day-old vaccination with a vectored HVT + IBD vaccine (VAXXITEK® HVT+IBD)

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Assessment of the immune response in broilers and pullets using two ELISA kits after in ovo or day-old vaccination with a vectored HVT + IBD vaccine (VAXXITEK[®] HVT+IBD)

By courtesy of



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In young chicks, a rapid onset of immunity post vaccination is critical, because, after the decline of maternally derived antibodies (MDA), the

birds are at risk of infection. However, high MDA titres interfere with early vaccination against Infectious Bursal Disease (IBD) using classical modified-live vaccines (MLVs) (intermediate and intermediate plus).

The development of an active antibody response to vaccination was evaluated using two ELISA kits (PROFLOK[®] IBD Ab test and an “improved” kit, PROFLOK Plus IBD Ab test) in commercial flocks of broilers and pullets vaccinated with different IBD vaccines.

An immunity gap was observed between 3 and 5 weeks of age in chickens vaccinated with MLVs (including an immune complex vaccine). In contrast, an early active immune

response was detected from 2 weeks of age in chicks vaccinated with vHVT13 (VAXXITEK[®] HVT+IBD) *in ovo* or subcutaneously at one day of age, using the PROFLOK Plus IBD Ab test, confirming that MDA do not interfere with this vaccine.

The combined use of both ELISA kits enables differentiation between chickens vac-

inated with VAXXITEK HVT+IBD from birds naturally infected or vaccinated with IBD MLVs: VAXXITEK HVT+IBD vaccinated chickens had low antibody titres using PROFLOK IBD Ab test and high antibody titres (usually >4000) using PROFLOK Plus IBD Ab test after 3 to 4 weeks of age. In contrast, chickens infected by IBDV or vaccinated with MLVs had high antibody titres with both tests after 6 weeks of age.

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Introduction

Generalities

Infectious Bursal Disease (IBD) or Gumboro disease is caused by a small, non-enveloped double-stranded RNA virus, belonging to the *Birnaviridae* family. Although it was first recognised more than 40 years ago, in 1962 in Gumboro, Delaware, USA, the disease still causes significant economic losses in the poultry industry worldwide. The bursa of Fabricius is the main target of the IBD virus (IBDV), which induces an immunosuppression in young birds, especially



between 3 and 6 weeks of age. In addition to the direct economic losses of the clinical disease, the damage caused to the immune system results in lowered resistance to other infectious agents and a poor response to commonly used vaccines.

The disease is highly contagious in young chickens. The economic impact of the disease depends upon the IBDV strain, the chicken breed (White Leghorns are more susceptible than broilers and brown-egg layers), concurrent primary or secondary pathogens, as well as environmental and management factors (Müller *et al.*, 2003). At the end of the 1980s, antigenic variants in the United States of America and very virulent (vv) IBDV strains in Europe (antigenically similar to the “classical strains”) emerged in vaccinated flocks and rapidly spread all over the world. Infection with classical virulent strains or variant American strains results in high morbidity and usually low mortal-





ity, whereas vvIBDV strains can cause up to 90% of mortality in layer-type birds (reviewed by Saif, 1998; van den Berg, 2000; Müller *et al.*, 2003).

There are two serotypes of IBDV, designated 1 and 2. Viruses of both serotypes naturally infect chickens and turkeys, but the disease is recognised only in chickens and only serotype 1 viruses are pathogenic. VP2 and VP3 are the major structural proteins, forming the outer and inner capsid of the virus, respectively (Böttcher *et al.*, 1997; reviewed by Saif, 1998; van den Berg, 2000; Müller *et al.*, 2003). The antigenic site responsible for the induction of neutralising antibodies is within a minimal region, called the variable domain of VP2, and is highly conformation-dependent (Becht *et al.*, 1988; Schnitzler *et al.*, 1993). This site is also responsible for serotype specificity. Conversely, VP3 is a group-specific antigen that is

recognized by non-neutralising antibodies, which may cross-react with both serotypes (Oppling *et al.*, 1991).

Vaccines

Because of the economic significance of the disease and the high prevalence of IBDV, vaccination has remained essential. Although cellular immunity may play some role, neutralising antibodies are essential for the protection against IBDV. MDA provide the young chicks with an excellent passive protection against infection. Inactivated oil-adjuvanted vaccines are commonly used to induce high antibody titres in breeders prior to the onset of lay, which will then be passively transmitted to the offspring. With the emergence of





vvIBDVs, it was no longer possible to rely solely on passive immunity to protect broilers, and a live vaccination was reinforced. Different MLVs containing classical or variant viruses are commercially available, and are classified according to their degree of attenuation as “mild”, “intermediate”, “intermediate plus” and “hot” IBD vaccines (reviewed by Saif, 1998; van den Berg, 2000; Müller *et al.*, 2003). The major problem with active immunisation is the interference with maternally derived antibodies (MDA) which may neutralise the vaccine. Thus the timing of IBD vaccine administration is crucial. The Deventer formula provides a useful tool to estimate the optimal vaccination timing for a specific flock, based upon the level and half-life of MDA, the age of the chickens at sampling, the genetic background of the chickens, and the IBD vaccine strain (de Witt, 2001). Two large field studies involving several broiler flocks with MDA have clearly shown the importance of vaccination at the optimal time point, which was estimated using the Deventer formula. If birds had received the intermediate IBD vaccine more than 1 day before the calculated optimal vaccination date, the humoral response was delayed or non-detectable until slaughter (Block *et al.*, 2007). Mild and intermediate vaccines are safer, in that they cause less bursal damage, than “hot” vaccines, but have a poor efficacy in the presence of MDA and against vvIBDVs. In contrast, less attenuated strains (“intermediate plus”

and “hot” vaccines) can overcome higher levels of MDA, but they may cause more severe lesions in the bursa follicles, resulting in immunosuppression. These strains are not recommended for chickens younger than 10 days of age. Alternatively, other types of vaccines have been developed which are less sensitive to the interference of passive immunity. An immune complex (IC) vaccine is used for *in ovo* or for s.c. day old vaccination, in which the “intermediate plus” vaccine virus is complexed with antibodies. It has been suggested that the IC is taken up by follicular dendritic cells (macrophages) where the virus resides until the drop of MDA. Vectored viral vaccines expressing proteins of IBDV have also been described as potential IBD vaccines, using vectors such as fowl pox virus, turkey herpes virus (HVT) (Darteil *et al.*, 1995), fowl adenovirus, Marek’s disease virus and Semliki Forest virus (reviewed by Saif, 1998; van den Berg, 2000; Müller *et al.*, 2003).

The HVT vector vaccine expressing the IBDV VP2 gene, designated vHVT013-69 (vHVT13) (VAXXITEK HVT+IBD), has the advantage of combining both enhanced safety and efficacy. Its efficacy has been demonstrated against Marek’s disease (the parental strain of HVT has been widely used in vaccination against Marek’s disease) as well as different strains of IBDV, including classical, very virulent and American variant IBDV strains. vHVT13 can be administered either *in ovo*

(3 days before hatching) or by the subcutaneous route in 1-day old chicks, in the presence of high titres of MDA (reviewed by Bublot *et al.*, 2007).

Serological tests

IBDV-specific antibodies can be measured by the virus neutralisa-

tion (VN) assay, ELISA or agar gel precipitation (AGP) test. The VN is the only test which can differentiate IBDV isolates into antigenic serotypes and subtypes. VN titres accurately reflect the protection of chickens to IBDV (Macreadie *et al.*, 1990; Fahey *et al.*, 1991; Al Natour *et al.*, 2004).



The AGP test is rapid but insensitive, and detects antibodies against primarily group-specific soluble antigens (Lukert and Saif, 1997). ELISA tests are the most commonly used, because they are economical, simple, rapid, reproducible and adapted to large scale use. Several ELISA kits are commercially available; they use different types of IBDV antigens, such as tissue-culture derived antigens, baculovirus expressed VP2, VP3, VP4 antigens (Jackwood *et al.*, 1999), or bursal-derived antigen.

Material and methods

a. Experimental design

A total of 18 commercial flocks of broilers in different countries (Italy, France, and Hungary) were used. For each trial, vHVT13 (VAXXITEK HVT+IBD) was compared to another IBD vaccine (intermediate, intermediate plus or IC vaccine). For each trial, birds of the same flock were randomly allocated to two groups and kept in different houses after vaccination. The vaccines were administered according

to the manufacturer's recommendations, either in drinking water or by subcutaneous (SC) route. vHVT13 was administered *in ovo* 3 days before hatching or by the SC route to 1-day-old chicks.

Pullet flocks from breeders vaccinated with IBD MLVs only, were also vaccinated with intermediate IBD vaccine or vHVT13 (SC, at 1 day of age) and maintained in field conditions.

In addition, an experiment was carried out in pullets in a contained environment (isolators). One-day old chicks were allocated to six groups. Two groups received by oral drop intermediate vaccines at 17 and 25 days of age, one group received by oral drop an intermediate plus vaccine on day 17, and one group was vaccinated subcutaneously with vHVT13 at 1 day of age. Two groups remained unvaccinated. All groups were challenged with a vvIBDV (77165 strain) (Martin *et al.*, 2007) on day 42, except for one unvaccinated group. In all groups, blood samples (10 to 25 samples/group) were randomly collected at regular intervals starting at day-old, and antibody titres were measured using two ELISA kits.

Figure 1a

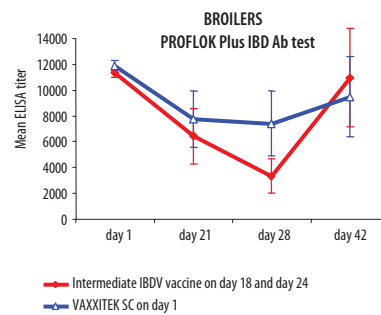
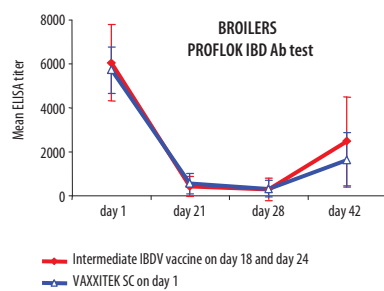


Figure 1b

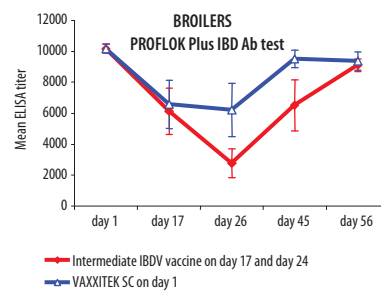
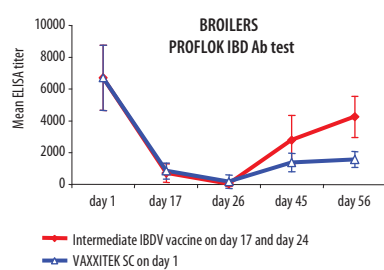


Figure 1 – ELISA IBDV mean antibody titres (\pm standard deviation) in broilers vaccinated with intermediate vaccines or VAXXITEK HVT+IBD (*in ovo* [Figure 1a] or SC [Figure 1b]).

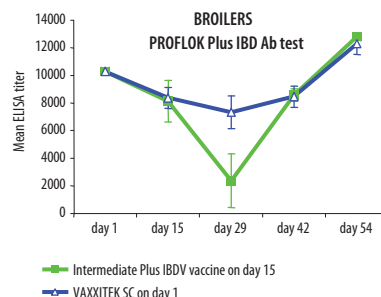
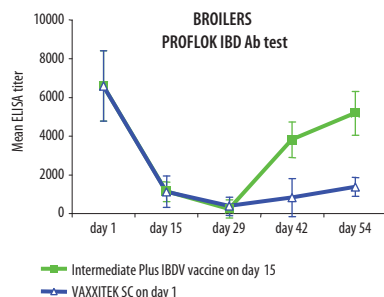


Figure 2 – ELISA IBDV mean antibody titres (\pm standard deviation) in broilers vaccinated with an intermediate plus vaccine or VAXXITEK HVT+IBD (SC on day 1)

b. ELISA kits for the detection of IBDV antibodies

Two ELISA kits have been used: the PROFLOK IBD Ab test kit and an “improved” kit, the PROFLOK Plus IBD Ab test kit (Synbiotics, San Diego, CA).

Both kits are indirect ELISAs and recognise antibodies to both classical and variant strains of IBDV. The principle of both tests is



similar. Specific IBDV antibodies in the test serum form an antigen-antibody complex with the IBD coated antigen bound to the plate. The antigen-antibody complex is detected by a peroxidase conjugate [anti-chicken IgG (H+L)]. The difference between the two tests lies in the nature of the IBDV antigen coated on the plates. PROFLOK IBD Ab test uses an antigen derived from a classical strain grown in tissue culture. PROFLOK Plus IBD Ab test uses a native bursal derived classical strain antigen. PROFLOK Plus IBD Ab test allows a more accurate detection of IBDV protective VP2 antibodies. This improved test is more sensitive than the classical test and highly correlated to VN test (Lamichhane *et al*, personal communication).

The threshold of positivity is set at a titre of 554 for the PROFLOK IBD Ab test and at 1002 for the PROFLOK Plus IBD Ab test.

c. Presentation of the data

Data were graphically presented using either mean curves with standard deviations or box-and-whisker plots. A box-and-whisker plot shows the distribution of a set

of data along a number line, dividing the data into four parts using the median and quartiles. Outliers (unusually small or large values) are also indicated.

Results

a. Antibody response kinetics

In broilers. Several trials were performed. The antibody response kinetics were comparable between trials. Therefore, only the results of a representative trial are shown, for each of the vaccines.

After an initial decrease of MDA during the first 2 weeks of age, the mean ELISA antibody response depended upon the type of vaccine administered (*Figures 1 to 3*):

Using the classical IBD test, antibody titres remained low in chick-

ens from 15 days to 21/28 days of age, then rose. Using this test, the seroconversion observed was lower in the vHVT13 group than in the other vaccinated groups.

Using the IBD Plus ELISA test, the mean antibody titres in the vHVT13 groups remained high (>6000) from day 15/17 to day 26/29. In contrast, the mean titres in birds vaccinated with the classical MLVs dropped (<4000) until day 21/28, and then increased at day 42/45, suggesting an immune gap in these birds.

In pullets. The pattern of antibody responses after vaccination in the field with intermediate and vHVT13 vaccines was very similar in broilers and pullets (compare *Figures 1 to Figure 4*).

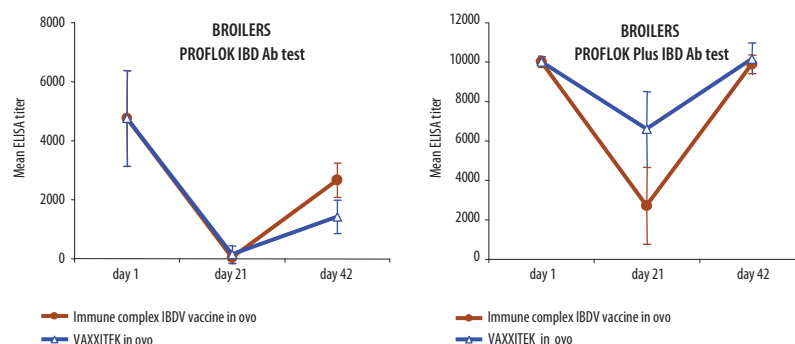


Figure 3 – ELISA IBDV mean antibody titres (\pm standard deviation) in broilers vaccinated with an Immune Complex vaccine or VAXXITEK HVT+IBD (in ovo)

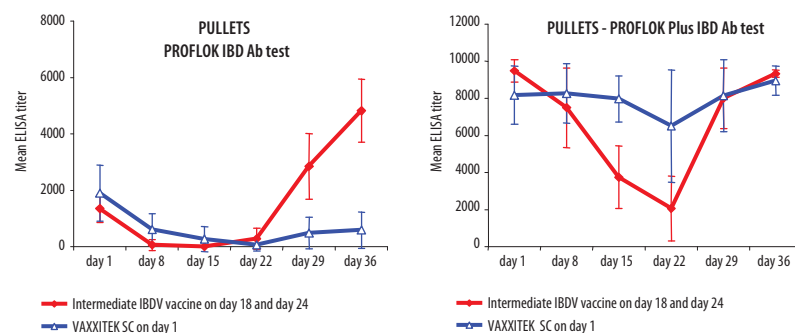


Figure 4 – ELISA IBDV mean antibody titres (\pm standard deviation) in pullets vaccinated with an intermediate vaccine or VAXXITEK HVT+IBD (SC on day 1)

b. Experimental vaccination/challenge trial in pullets

Before challenge, a decline of antibody titres was observed in all groups, using both the classical and IBD Plus ELISA tests, except in the vHVT13 vaccinated group. Using the IBD Plus ELISA test, antibody titres from the vHVT13 vaccinated group remained high and stable. The lack of detectable antibody response in the modified-live vaccinated groups on day 42 was probably due to the interference with MDA at the time of vaccination.

After challenge, with both ELISA tests, a marked increase in antibody titres was observed in all groups, except in the vHVT13 vaccinated group which had only a slight increase (Figure 5).

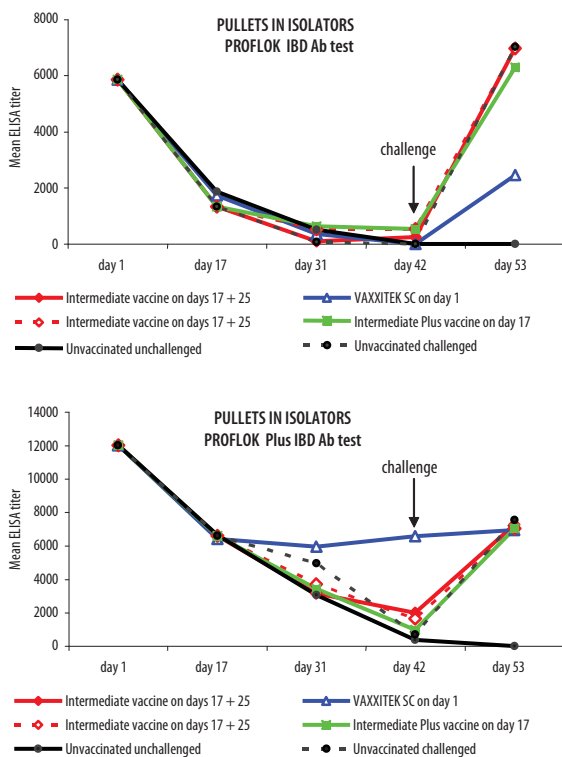


Figure 5 – ELISA IBDV mean antibody titres in pullets vaccinated or not with intermediate, intermediate plus vaccines or VAXXITEK HVT+IBD (SC on day 1) and maintained in isolators. All groups were challenged on day 42, except for one group of unvaccinated pullets.

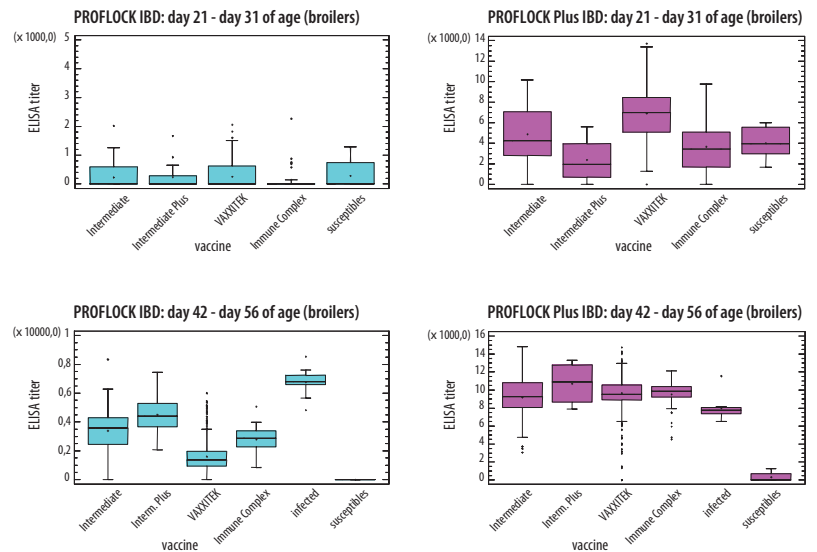


Figure 6 – ELISA IBDV antibody titre distribution (box and whisker plots) in broilers of different status during two periods of life

Based on clinical signs following challenge, full protection was observed in vHVT13 vaccinated birds and a partial protection in birds vaccinated with intermediate or intermediate plus vaccines.

c. Antibody response according to the vaccination status during two periods of life

All antibody results obtained from day 1 to day 56 in our field trials in broilers vaccinated with different vaccines were pooled (n=2472 samples). As no field data from susceptible (non-vaccinated and not infected) and IBDV infected broilers were available, we used the antibody titres from pullets challenged or not challenged in isolators (n=200 samples). The antibody responses obtained at two periods of age (from day 21 to day 31, and from day 42 to day 56) are presented in Figure 6. During the 21-31 day period, relatively low antibody titres were detected using the classical IBD ELISA test in all

groups, whereas, using the IBD Plus ELISA test, higher antibody titres were observed in the vHVT13 group. During the 42-56 day period, antibody titres measured with the classical IBD ELISA test were lower in the vHVT13 group than in the other vaccinated and infected groups, and high antibody titres were observed in all vaccinated and infected groups using the IBD Plus ELISA test (Figure 6).



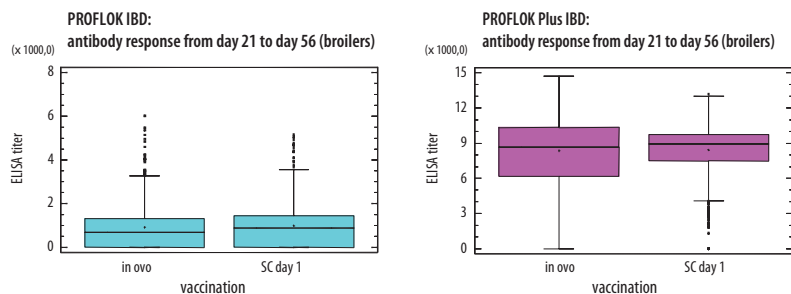


Figure 8 – ELISA IBDV antibody titre distribution (box and whisker plots) in broilers after vaccination with VAXXITEK HVT+IBD *in ovo* or at 1 day of age

A strategy could then be routinely applied using both ELISA kits at each period of life to differentiate between chickens vaccinated with vHVT13 from susceptible, infected chickens or vaccinated with classical vaccines (Figure 7).

d. Global antibody response in vHVT13 vaccinated groups

Vaccination with vHVT13 *in ovo* or SC at 1 day of age. In broilers, the antibody response from day 21 to day 56 of age was similar after vaccination with vHVT13 whichever the route of immunisation (*in ovo* [n=370] or subcutaneous (SC) at 1 day of age [n=700]) (Figure 8).

Distribution of antibody titres measured by IBD Plus ELISA test. Among the 1101 samples collected in the different trials in broilers between day 21 and day 56 of age, antibodies were not detected in four chickens (0.4% of samples).

Antibody titres were above 4,000 in 93% of the samples (Figure 9). Mean antibody titre was 8,345 (range 0 to 14,737) and the coefficient of variation was 30%.

Assessment of the effectiveness of vaccination with vHVT13.

Sera were collected in one flock of broilers vaccinated with vHVT13 at 1 day of age. Mean antibody titres were abnormally low, close to 2,500 on day 30 of age using the IBD Plus ELISA kit (data not shown). An audit was formerly performed at the hatchery on 800 chicks out of 90,000 using a blue dye. It revealed that more than 20% of the chicks had not been properly vaccinated, because of an excessive working speed, in conjunction with some technical problems with the vaccinating machines.

		PROFLOK Plus IBD Ab test	
		+++	+ or -
PROFLOK IBD Ab test	+++	not observed*	unlikely
	+ or -	VAXXITEK vaccinated birds	MLV vaccinated or susceptible birds

* in our data, but may occur in case of early IBD

		PROFLOK Plus IBD Ab test	
		+++	+ or -
PROFLOK IBD Ab test	+++	infected or MLV vaccinated	unlikely
	+ or -	VAXXITEK vaccinated birds	susceptible birds

Figure 7 – Antibody response according to the status during two periods of life

Discussion

The presence of IBDV MDA in chicks is a major problem, because they may interfere with classical IBD MLVs, such as the intermediate, intermediate plus and immune complex vaccines. In presence of

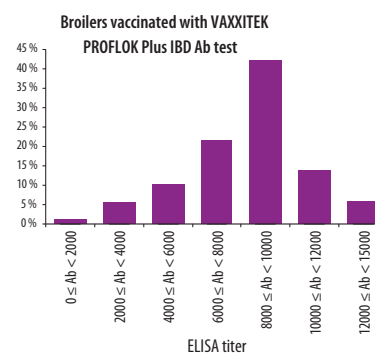


Figure 9 – ELISA IBDV antibody distribution in broilers from day 21 to day 56 of age after vaccination with VAXXITEK HVT+IBD

MDA, active immunisation with classical vaccines remains hazardous. After the decline of MDA, birds which do not respond to vaccination are susceptible to IBDV infection. Our ELISA results illus-



trate the immunity gap observed in groups vaccinated with MLVs (including immune complex vaccine) between approximately 15 and 28 days of age. In contrast, using a specific IBD Plus ELISA test correlating with neutralising antibody test, an active and strong anti-VP2 response was observed from day 15 of age in chickens vaccinated with vHVT13 (VAXXITEK HVT+IBD). Hence, no immunity gap occurred during the 3 to 5 weeks of age period, the most critical for IBDV field challenge. This earlier response can be obtained because vHVT13 efficacy

is not affected by the presence of high levels of MDA, and can thus be administered either *in ovo* or at 1 day of age. The cell-associated nature of the vHVT13 vaccine (consisting of vHVT13-infected chicken embryo fibroblasts), the lack of expression of VP2 on the surface of infected cells or of HVT vector virus, and the mode of replication of the HVT vector probably all contribute to the ability of this vaccine to overcome MDA (Bublot *et al.*, 2007). Anti-VP2 antibodies induced by vHVT13 are protective as already described previously (Goutebroze *et al.*, 2003; Bublot *et*

al., 2007) and as confirmed by vaccination/challenge experiments in pullets maintained in isolators and in broilers.

Interestingly, antibody response detected by the “classic” IBD ELISA test was low in vHVT13 vaccinated groups, and relatively high in MLVs vaccinated groups. This may be explained by the nature of the antigen used for the detection of antibodies. The “classic” IBD ELISA uses an antigen of classic cell-culture origin, and the IBD Plus ELISA test uses a bursal grown virus. It has occasionally been described, that tissue culture adapted virus has changed antigenicity. For example, the monoclonal antibody (Mab) 21 recognised all the vvIBDVs but did not bind to tissue culture-adapted virus (Mengel-Whereat, 1995; Vakharia *et al.*, 2000). Neutralising Mab 8 detected conformation epitopes of IBDV and recognised only bursal-grown virus but not cell culture-adapted virus (Lamichhane *et al.*, personal communication). The amino acids involved in cell culture adaptation, virulence and cell tropism of IBDV have been mapped in VP2 hypervariable region (also designated P domain) by reverse genetics (Boot *et al.*, 2000; Brandt *et al.*, 2001; Letzel *et al.*, 2007; Lim *et al.*, 1999; Mundt, 1999; van Loon *et al.*, 2002). Considering that cell culture adaptation may change the antigenicity of VP2, the new ELISA, PROFLOK Plus IBD Ab test, was developed by coating ELISA plates with native IBDV antigen of bursal origin. This improved test is



more sensitive than the classical test and highly correlated to VN test (Lamichhane *et al.*, personal communication).

Our results showed that vHVT13 induced a similar active immune response in broilers and pullets. The antibody response was comparable in vHVT13-immunised broilers whatever the route of administration, *in ovo* or subcutaneous (Figure 8).

Antibody profiling of flocks is very useful to assess the effectiveness of vaccination or the persistence of antibodies. After vaccination with vHVT13, antibody titres were usually higher than 4000 throughout the 21-56 day period, using the ELISA Plus test (Figure 9). The coefficients of variation of vHVT13 vaccinated groups were usually satisfactory ($\leq 30\%$), suggesting a homogeneous immune response. The rate of non responders was low (0.4% of all birds tested). A higher number of non-responders, observed in one farm, may reflect a problem at the time of administration of the vaccine. Serological monitoring is also critical for the diagnosis of a field infection, which could have dramatic consequences especially with vvIBDV strains. The use of vHVT13 vectored vaccine makes it possible to differentiate the susceptible, immunised or field-challenged chickens. With the combined use of the two IBD ELISA tests, the field situation can be more efficiently monitored by discriminating between vHVT13-induced antibodies (anti-VP2 only, detected by the IBD Plus ELISA

test) and IBDV infection-induced antibodies (including anti-VP3 detected by the classical IBD test). High antibody titres were detected in IBDV infected chickens or chickens vaccinated with MLVs, using both tests. In contrast, vHVT13 vaccinated chickens had high antibody titres with the IBD Plus ELISA test, and low titres with the classical IBD ELISA test (Figure 7). The current IBD ELISA tests do not allow differentiation between infected chickens and chickens vaccinated with MLVs. This is a problem, in particular when bursal lesions are observed. In contrast to vHVT13, classical vaccines – even intermediate strains – may also induce bursal lesions, and the only way to distinguish vaccinates from infected birds is to amplify and sequence the RNA of the IBDV strain responsible for the bursal lesions (Block *et al.*, 2007; Bublot *et al.*, 2007).

In conclusion, an early active immune response can be induced in chickens after *in ovo* or day-old vaccination with vHVT13 even in the presence of high MDA. The immunity gap observed with all MLVs vaccinated birds tested between 3 and 5 weeks of age was not observed in vHVT13-vaccinated birds. The induction of antibodies by vHVT13 can be measured using a specific IBD Plus ELISA kit that correlates with VN test. The combined use of the classic and IBD Plus ELISA kits makes it possible to differentiate birds vaccinated with vHVT13 from birds infected or vaccinated with MLVs.



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