

COMPARISON OF CVI AND HVT VACCINE STRAINS REPLICATION IN FEATHER TIPS IN DIFFERENT MAREK'S DISEASE VACCINATION PROGRAMS

COMPARAÇÃO DA REPLICAÇÃO DA ESTIRPE VACINAL CVI E HVT NO FOLÍCULO DA PENA EM DIFERENTES PROGRAMAS VACINAIS PARA A DOENÇA DE MAREK

E. C. MUNIZ^{1*}, D. T. ITO², L. A. RODRIGUES², G. B. C. SALLES¹, A. L. KRAIESKI¹,
D. V. NASCIMENTO JUNIOR¹, A. J. LIMA NETO¹, A. S. K. FONSECA³

SUMMARY

Marek's disease is an important neoplastic disease in birds caused by a serotype 1 specific herpesvirus; it is controlled by vaccination. In commercial breeders and layers in Brazil, current vaccination programs use the combination of attenuated or non-pathogenic strains of the HVT virus (turkey herpesvirus - serotype 3) and CVI 988 (Rispens - serotype 1). The combination of serotype 3 and 1 it has been an important and effective control strategy through the vaccination of long-lived birds. In addition, more recently the recombinant rHVT strain (vectorized vaccines) has been used in some vaccine programs. This study's main objective was to compare CVI and HVT components' replication in feather tips in three different *Marek's disease* (MD) immunoprophylactic programs (T01 – program A, T02 – program B and T03 – program C). Quantification of these two vaccine strains was performed by real-time PCR in samples collected at the ages of 14, 21, and 28 days. At 14 days, mean of log[cvi] in program B was significantly higher than C ($p<0.05$). At 21 and 28 days, mean of log[cvi] of program C was significantly lower than A and B ($p<0.05$). For mean of log[hvt], at 28 days, program B was significantly higher than A ($p<0.05$). For proportion of positives, at 14 days, program B had 2.7 times more risk to be positive in CVI than program C ($p<0.001$). At 21 days, program B had 1.7 times more risk to be positive in CVI than program C ($p=0.005$). For HVT, at 28 days, program B had 3.2 times more risk to be positive than program A ($p=0,009$). Results showed significant differences between the treatments evaluated. In general the conventional combination Marek's vaccine containing CVI+HVT (program B) showed higher replication rate and percentage of vaccine coverage than the programs with rHVT vector vaccines (program A and C).

KEY-WORDS: Marek's disease, vaccination, conventional vaccine, vector vaccine and long-lived birds.

RESUMO

A doença de Marek é uma importante doença neoplásica das aves causada por um herpesvírus específico do sorotipo 1 e seu controle se faz por vacinação. Em reprodutoras e poedeiras comerciais do Brasil, os programas de vacinação utilizam a combinação de estirpes atenuadas ou não patogênicas do vírus HVT (*turkey herpesvirus* - sorotipo 3) e CVI 988 (*Rispens* - sorotipo 1). A combinação do sorotipo 3 e 1 tem sido uma importante e efetiva estratégia de controle para aves de vida longa. Além disso, mais recentemente a fração rHVT recombinante (vacinas vetorizadas) vem sendo utilizada em alguns programas vacinais. O objetivo deste estudo foi comparar a replicação das frações CVI e HVT no folículo da pena em três programas imunoprofiláticos distintos (T01 – programa A, T02 – programa B e T03 – programa C). A quantificação das duas estirpes vacinais foi realizada por PCR em tempo real nas amostras colhidas nas idades de 14, 21 e 28 dias. Aos 14 dias, em média, log[cvi] do programa B foi significativamente maior do que C ($p<0.05$). Aos 21 e 28 dias, a média do log[cvi] do programa C foi significativamente menor do que A e B ($p<0.05$). Para log[hvt], aos 28 dias, a média do programa B foi significativamente maior do que A ($p<0.05$). Para proporção de positivos, aos 14 dias, o programa B teve 2,7 vezes mais risco de ter positivos no CVI do que C ($p<0.001$). Aos 21 dias, o programa B teve 1,7 vezes mais risco de ter positivos no CVI do que C ($p=0.005$). Para HVT, aos 28 dias, o programa B teve 3,2 vezes mais risco de ter positivos do que A ($p=0,009$). Os resultados obtidos evidenciam diferenças significativas entre os tratamentos. De maneira geral a vacina convencional de Marek combinada com o CVI e HVT (programa B) apresentou maior taxa de replicação, velocidade e percentual de cobertura vacinal do que os programas compostos com vacinas vetorizadas com rHVT (programa A e C).

PALAVRAS-CHAVE: Doença de Marek, vacinação, vacina convencional, vacina vetorizada e aves de vida longa.

¹ Zoetis Indústria de Produtos Veterinários Ltda – São Paulo-SP/Brazil

² Hisex Genetics Ltda – Salto-SP/Brazil

³ Simbios Biotecnologia – Cachoerinha-RS/Brazil

*Corresponding author: Eduardo Correa Muniz, Phone number: +55 47 99626-2463. Av. Dr. Chucris Zaidan, 1240 – Morumbi Corporate – Ed. Diamond Tower, 4º andar – São Paulo/SP - Brazil | CEP: 04709-111. E-mail: eduardo.muniz@zoetis.com

INTRODUCTION

Marek's disease (MD) is caused by a herpesvirus affecting birds. It is characterized by lymphoproliferative neoplasms in peripheral nerves and organs such as gonads, iris, viscera, muscles, and skin (Witter, 1997). This disease has accounted for economic losses worldwide as it causes mortality, immunosuppression, and loss of performance in birds. It nearly devastated the poultry industry in the 1960s before HVT-based vaccines were developed. This was the first successful case of controlling a disease caused by an oncogenic virus (Witter, 1998). Thus, vaccines have been used to control Marek's disease in commercial birds since 1970 to minimize the resulting economic losses (Baigent et al., 2006).

Marek's disease viruses (MDV) are cell-associated. They belong to the family Herpesviridae, subfamily Alpha-herpesviridae, and genus *Mardivirus* (Fauget et al., 2005). There have been three serotypes classified: serotype 1 (MDV1), serotype 2 (MDV2), and serotype 3 (MDV3 or turkey herpesvirus, HVT). MDV1 is oncogenic and is the etiologic agent of Marek's disease. The other two serotypes (MDV2 and MDV3) are non-oncogenic and are used in monovalent or bivalent live MD vaccines or even associated with attenuated MDV1 strains (such as CVI988) (Witter & Schat, 2003).

Current vaccination programs use the combination of attenuated or non-pathogenic HVT strains (serotype 3) and CVI 988 (Rispen, serotype 1) in Brazilian commercial breeders and layers. The combination of serotype 3 and 1 has been an important and effective control strategy by means of vaccination of long-lived birds since it provides better protection against field challenges (Davison & Nair, 2005). The CVI component is considered the most effective strain for protection against the highly virulent Marek's viruses (Gimeno et al., 2015a).

More recently, with the broad development of vector vaccines, different programs have been adopted for commercial breeders and layers using a recombinant HVT component (rHVT) also aiming to protect against Gumboro disease (also known as infectious bursal disease - IBD). These vectorized vaccines are developed by genetic engineering where an insert of a pathogen is incorporated into the vector virus. In that case, an immunizing portion of the Gumboro's DNA virus is inserted into Marek's HVT vector virus (rHVT-IBD). Thus, the vectorized vaccine has the benefit of immunizing against both diseases. On the other hand, when using conventional vaccination programs against Marek's Disease, the producer must associate another vaccine to protect Gumboro. An alternative to compose the conventional program has been to use the HVT and CVI viruses with immunocomplex vaccines against Gumboro that can also be administered in the hatchery. The immunocomplex vaccines used in these programs consist of an attenuated strain of the Gumboro virus covered by antibodies.

This study's objective was to compare the replication rate of HVT and CVI components in different vaccination programs commonly used to control Marek's disease in commercial layer or breeders in Brazil.

MATERIAL AND METHODS

Birds and experimental design

A total of 300 1-day-old female birds, Hisex White breed (provided by Hendrix Genetics company) were housed in 3 identical cages in the poultry house at the experimental farm Professor Hélio Barbosa, part of the Federal University of Minas Gerais (UFMG). Pre-housing disinfection procedures were performed to prevent the spread of any infectious or vaccine agents to the trial birds. The birds were raised on wood shaving litter, with tubular feeders, bell-type hanging drinkers, and heat lamps. The birds were provided with mash feed *ad libitum* throughout the trial following Hendrix Genetics' recommendations. The animals were divided into three groups that contained 100 birds each according to the vaccination programs usually adopted against IBD and Marek's disease as shown in the Table 1.

This protocol was submitted to the Committee on Ethics in the Use of Animals (CEUA) in accordance with standards issued by the National Council on Animal Experimentation (CONCEA). It was approved and registered under # 009/20.

Vaccine and vaccination

Each bird received a post-hatch subcutaneous dose of the vaccine at the hatchery. The volume injected per bird was 0.2 mL in the middle third of the back of the neck. All birds received one dose of the same mild strain fowl pox vaccine in addition to treatment vaccines. The vaccines used were commercially available products routinely used in the bird hatchery without any prior orientation or pre-selection of the batches used. All products used in this study are licensed and registered for use and are commonly used in commercial hatcheries. The titers of each vaccine used is shown in the Table 1. Program A did not provide the titers of the HVT and CVI strains separately. The available titer for the program in T01 refers to the total of the two strains.

The commercial products used in this study belonged to three different suppliers. Although the characteristics of the products in T01 and T03 are similar (vectorized vaccine rHVT-IBD), they were from different laboratories.

Sampling

Feathers randomly selected from 20 birds/group were sampled at the ages of 14, 21, and 28 days. The sampling was performed by one single person who changed gloves after completing collection in each group in order to avoid any possible cross contamination between treatments. After collections at different ages, the birds were returned to their

respective group with 100 birds per treatment. The samples consisted of 10 selected pulp-rich feathers collected on each bird's axillary tract. They were stored in tubes identified by the color of the corresponding treatment. These samples were sent to Simbios laboratory for quantitative testing of both CVI and HVT component of MDV vaccine strain.

Throughout the trial, samples were identified only by colors to prevent knowledge of the treatments' composition (blind testing). Feather tips have already been well established as an excellent source for detecting and quantifying MV (Baigent et al., 2005). It also has the advantage to be easily and individually sampled from birds by a non-invasive method.

Table 1 - Results of titers in different batches and vaccination programs used in this study.

Treatment	Vaccine used	Vaccine batch	Vaccine titers	Total titer (HVT + CVI)
T01 - Program A	HVT-IBD and Rispens CVI988	001/19	HVT component and CVI component, 12850 PFU/dose	12850 PFU/dose
T02 - Program B	Conventional combination vaccine containing HVT and CVI988	012/19	HVT component, 6160 PFU/dose	9840 PFU/dose
	Immune complex vaccine (V877005/18 strain)		CVI component, 3680 PFU/dose	
			2048 GMT	
T01 - Program C	HVT-IBD vector vaccine	017/19	HVT component, 7060 PFU/dose	13340 PFU/dose
	Rispens/CVI988 vaccine	001/19	CVI component, 6280 PFU/dose	

Laboratory analysis

The real-time polymerase chain reaction (PCR) assay was performed. It amplifies a target sequence of each component, using Meq specific genes for CVI (serotype 1) and sORF1 for HVT (serotype 3) (Handberg et al., 2001; Davidson & Borenshtain, 2003; Baigent et al., 2005). The results were expressed in the relative concentration of viral DNA by cell DNA from each bird (copies of vaccine virus/10,000 somatic cells).

DNA purification: Nucleic acids were extracted from field samples using NewGene Prep and NewGene Preamp reagents according to the manufacturer's instructions (Simbios Biotecnologia, Rio Grande do Sul, Brazil). Briefly, the feather pulps were added to 500 µL of lysis buffer (NewGene Prep) and then incubated at 60 ° C for 10 min. NewGene PreAmp reagents were used in the following steps: after centrifugation (10,000 x g, 1 min), the supernatant was transferred to a tube containing 20 µL of silica suspension. After vortexing and centrifuging (10,000 x g, 1 min), the pellet was washed with 300 µL of GuSCN-Tris buffer, followed by successive new washes, with 75% ethanol and absolute ethanol. The silica suspension was dried and the DNA eluted with 50 µL of elution buffer, of which 2.0 µL were used as a template in the real-time PCR reaction. The DNA was stored at - 20 ° C until use in real time PCR assays.

Real-time PCR for quantification of MDV-1, HVT (MDV-3): Quantification of MDV-1, HVT and chicken DNA was performed based on the real-time polymerase chain reaction (PCR) assay described by Islam et al. (2004). The procedure consists of amplifying a target sequence of specific Meq genes for MDV-1 (serotype 1), ORF1 for HVT (serotype 3) and collagen alpha₂ (VI) gene for chicken (to estimate the number of somatic cells). For each run of the assay, individual standard curves were generated using four to five decimal dilutions of MDV1, HVT and chicken DNA standards with known concentration. Samples that did not amplify or had a limit cycle value (Ct) below the lowest standard were reported as zero / negative. The results were expressed in the relative concentration of viral DNA by

cellular DNA of each bird (copies of the vaccine virus / 10,000 somatic cells). Real-time PCR assays were performed using StepOnePlus™ Real-Time PCR or Applied Biosystems QuantiStudio 1 Real-Time PCR System (Thermo Fisher Scientific). The cycling conditions were the same for both systems: 95 ° C for 3 min, followed by 40 cycles of 95 ° C for 15 s and 60 ° C for 60 s.

Statistical Analysis

Exploratory analysis was proceeded, initially with mean, standard deviation and normality tests for Log[cvi] and log[hvt]. Log[cvi] and log[hvt] are not normal distribution, so differences between vaccination programs were verified with Kruskal-Wallis test and Dunn as post-hoc. The differences of proportions of positives and negatives between vaccination programs were verified by qui-square test and the intensity of association was verified by relative risk estimation. Relative risk is a measure of ratio between exposed in one treatment and other treatment, evidencing when the differences are significant or not proportionality. All tests were considered significant when p<0.05 and analyses were proceeded in R environmental (R Core Team, 2019).

RESULTS

In Table 2 and Figures 1 and 2 are presented results of log[cvi] and log[hvt] in each phase and treatment. There were significant differences between vaccination programs in log[cvi] at 14, 21 and 28 days. At 14 days, program B was significantly higher than C in this variable, between AxB and AxC there was no differences. At 21 and 28 days, program C was significantly lower than A and B and between AxB there was no difference. For log[hvt], there was no significant differences between programs at 14 and 21 days. At 28 days, program B was significantly higher than A in this variable, between BxC and AxC there was no differences.

Table 2 - Descriptive statistics of log cvi and hvt for each treatment and p-values for differences on statistical tests.

		Log[cvi]			Log[hvt]		
		Mean	Standard deviation	p-value*	Mean	Standard deviation	p-value*
14 days	A	3.24 ab	2.34	0.001	0.28 a	0.76	0.971
	B	5.09 a	1.9		0.51 a	1.49	
	C	1.75 b	2.9		0.32 a	1.09	
21 days	A	5.81 a	1.71	<0.001	1.16 a	2.06	0.616
	B	6.60 a	1.31		1.73 a	2.36	
	C	2.40 b	2.22		1.80 a	2.09	
28 days	A	5.53 a	1.93	0.003	0.85 b	1.87	0.029
	B	5.79 a	1.73		2.25 ac	1.86	
	C	3.33 b	2.65		1.23 bc	1.64	

*p-value on ANOVA or Kruskal-Wallis comparing all vaccination programs; different minuscule letters corresponds to significant differences in multiple comparisons initiating by major to minor group

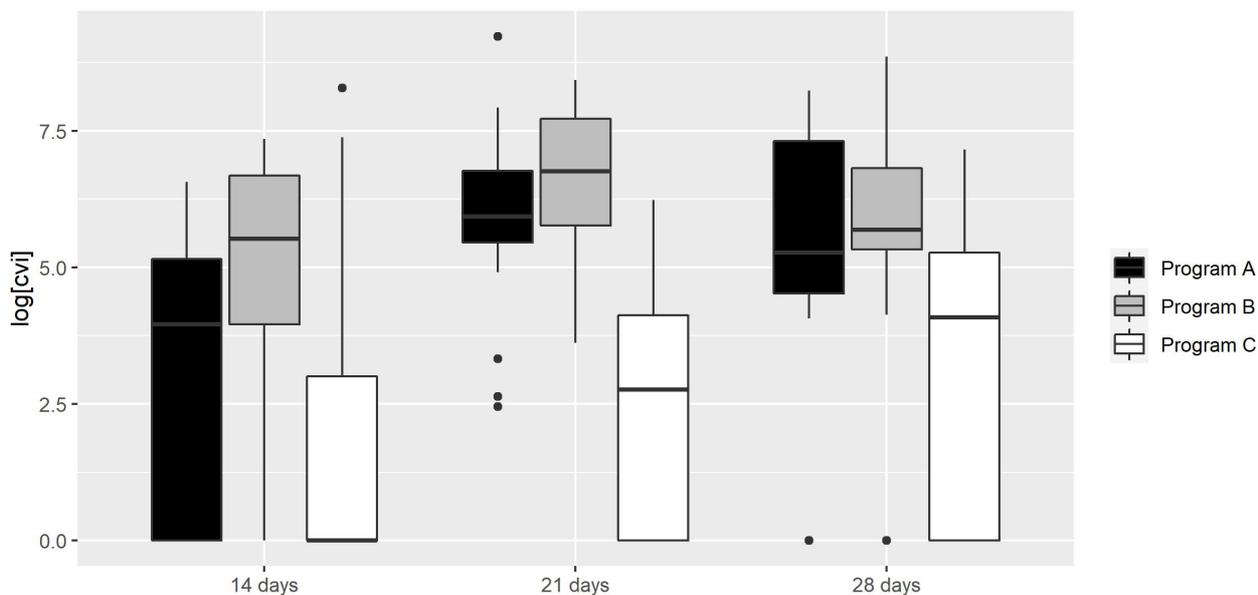


Figure 1 - Boxplot of log[cvi] by phase and vaccination program.

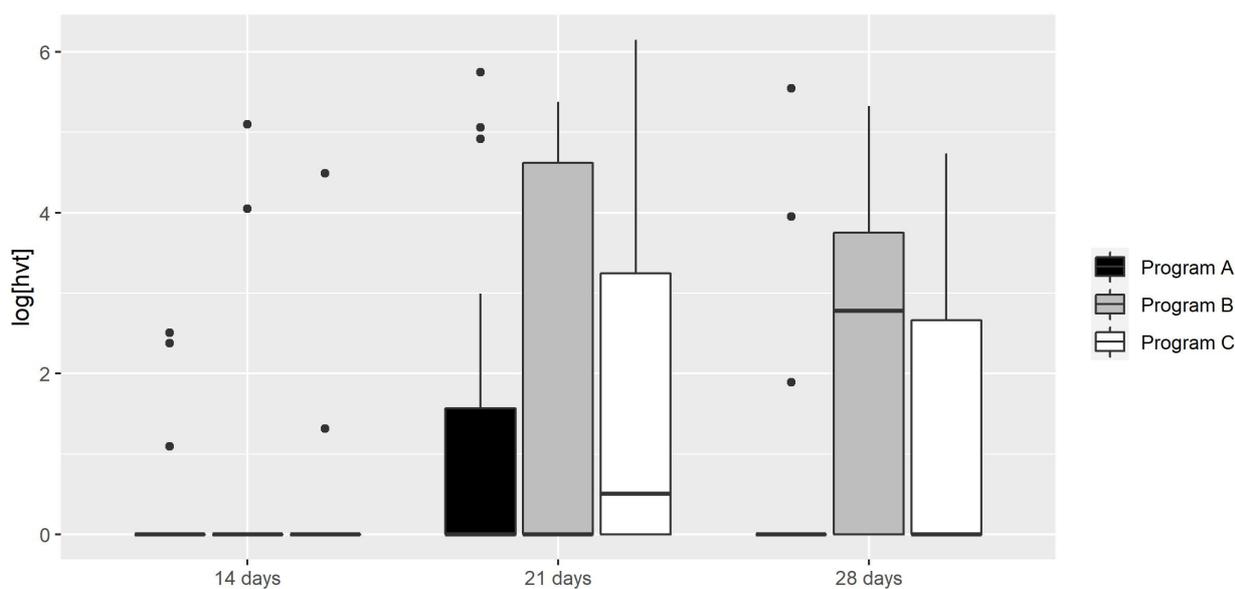


Figure 2 - Boxplot of log[hvt] by phase and vaccination program.

In Tables 3 and 4 are presented analysis of percent of positivity for CVI and HVT, respectively, in each program and each phase. In Table 3, at 14 days, program B had 2.7 times more risk to be positive in CVI than program C ($p < 0.001$) and between BxA there was no differences. At 21 days, program B had 1.7 times more risk to be positive in CVI than program C ($p = 0.005$) and

between BxA there was no difference. At 28 days, there was no differences between BxC or BxA.

In Table 4, at 14 or 21 days, there was no significant differences in proportions of positives between treatments for HVT. At 28 days, program B had 3.2 times more risk to be positive than program A ($p = 0.009$) e between BxC there was no significant difference.

Table 3 - Simple and relative frequencies of positivity in CVI in each treatment and phase.

CVI			Positive		Negative		RR (CI 95%)	p-value
			N	% group	N	% group		
14 days	Program	A	15	71.4%	6	28.6%	1.3 (0.9 – 1.8)	0.112
		B	19	95.0%	1	5.0%	Reference *	
		C	7	35.0%	13	65.0%	2.7 (1.5 – 4.9)	<0.001
21 days	Program	A	19	100.0%	0	0.0%	1.0 (0.9 – 1.1)	1.000
		B	20	100.0%	0	0.0%	Reference *	
		C	12	60.0%	8	40.0%	1.7 (1.2 – 2.4)	0.005
28 days	Program	A	19	95.0%	1	5.0%	1.0 (0.9 – 1.2)	1.000
		B	19	95.0%	1	5.0%	Reference *	
		C	14	70.0%	6	30.0%	1.4 (0.9 – 1.8)	0.096

*calculations comparing BxA e BxC

Table 4 - Simple and relative frequencies of positivity in HVT in each treatment and phase.

HVT			Positive		Negative		RR (CI 95%)	p-value
			N	% group	N	% group		
14 days	Program	A	3	14.3%	18	85.7%	0.7 (0.1 – 4.5)	0.675
		B	2	10.0%	18	90.0%	Reference*	
		C	2	10.0%	18	90.0%	1.0 (0.1 – 6.4)	1.000
21 days	Program	A	5	26.3%	14	73.7%	1.3 (0.5 – 3.5)	0.810
		B	7	35.0%	13	65.0%	Reference *	
		C	10	50.0%	10	50.0%	0.7 (0.3 – 1.5)	0.523
28 days	Program	A	4	20.0%	16	80.0%	3.2 (1.3 – 8.3)	0.009
		B	13	65.0%	7	35.0%	Reference *	
		C	8	40.0%	12	60.0%	1.6 (0.9 – 3.0)	0.205

*calculations comparing BxA e BxC

DISCUSSION

Lymphocytes are the main target of pathogenic MDV1. This makes it a highly contagious virus with lymphoproliferative, oncogenic, and immunosuppressive features. Replication of the pathogenic virus occurs in epithelial cells of the feather follicle's keratinizing layer and then highly infectious particles (dander) are released and propagated in the air (Calnek et al., 1970; Biggs, 1985). Quantification of MDV in feather tips, in dander contained in dust sampled in air hoods, in dust in poultry houses, and in lymphoid organs has been used to determine correlations among virulence, infectivity, shedding rate, and transmission (Islam & Walkden-Brown, 2007). This same technique is also useful in studies with vaccine strains of MDV1 (CVI988) (Rispen et al., 1972), MDV2 (Witter, 1987), and MDV3 (HVT), allowing comparison and monitoring of different vaccination programs and the behavior of each vaccine strain. Cho & Kenny (1975) demonstrated that, unlike MDV1 and MDV2, HVT does not spread rapidly among birds early in their life.

In this study, MDV quantification in feather tips by real-time PCR enabled to show significant differences in the replication rate of both CVI and HVT components among the treatments analyzed (Table 2). Similar results have already been described in other scientific studies that seek to understand the intensity and dynamics of replication of different Marek's vaccine strains, especially the effect of combining conventional CVI component with rHVT vector vaccines (Gimeno et al., 2015a, Gimeno et al., 2019). Several factors interfere in rHVT replication, such as the age at vaccination, and in-ovo vaccination leads to increased replication when compared to subcutaneous vaccination (Gimeno et al., 2016). Furthermore, the combination of rHVT and CVI results in a lower rHVT replication rate according to the CVI component dose used (Gimeno et al., 2019). Thus, although the combination of vaccines with different MVD serotypes results in better protection against MD, it also reduces the replication of each vaccine component used. This effect is more intense for rHVT.

In experimental studies with birds vaccinated against Marek's disease, it has been demonstrated that the optimum age for evaluating the vaccine take is

between 2 and 5 week post-vaccination when feather tips are pulp-rich for viral DNA extraction (Baigent et al., 2005). In Figures 1 and 2 it is possible to observe the results of samples submitted to qPCR quantification of CVI and HVT components of MDV. The objective of this analysis was to obtain an overall picture of the vaccination status by comparing different vaccination programs. At this point, when replication of the vaccine components has already reached their plateau, most birds are expected to have adequate protection. In this regard, T02 showed advantages in relation to the other treatments. However, a wide variability in MDV genome load detected among individuals was observed in all treatments. This variability among birds is also found in field research and has been justified by the fact that the birds might not have received an adequate vaccine dose (Baigent et al., 2006). Moreover, these variations have also been related to genetic factors, maternal antibody levels, and infections by other pathogens that may alter, delay, or even suppress the replication of MDV vaccine strain (Gimeno et al., 2005a).

Regarding vaccination coverage, it is very important to establish the correlation of the replication kinetics of the used vaccine's strain load with the protection against the Marek's disease's field virus. We can anticipate that the efficacy of the vaccine used is correlated with the load, the plateau, and the persistence of the vaccine virus in lymphoid tissue and in feather tips (Baigent & Davidson, 1999). The delay of Marek's vaccine in reaching its plateau is also an indication of the delay in establishing immunity which may be a disadvantage from the protection point of view because birds will be exposed to the virulent field strain in the first weeks of life (early challenge). (Gimeno et al., 2004). This represents an advantage of T02 when compared to the other treatments because replication of the CVI component clearly occurred faster than in T01 and in T03 (Figures 1 and 2).

It is known that all commercially available vaccines prevent mortality, the development of tumor lesions, but they are still not able to fully prevent infection and viral replication of the field challenge (Islam & Walkden-Brown, 2007). Even though all the mechanisms of immunity to Marek's disease are still unclear, we know that replication of vaccine strain induce a response by increasing the activity of natural killer cells, producing interferon-gamma and producing antigen-specific cytotoxic T lymphocytes that help eliminate infected cells 3 to 7 days after vaccination (Schat & Xing, 2000; Gimeno et al., 2015b). Whatever the precise protective mechanism, this study has confirmed significant differences when we compared the intensity of replication of the different vaccine strains and as a next step it would be pertinent to carry out additional studies with experimental challenge in order to determine the levels of protection among the different programs analyzed.

Besides, the results found also match published studies on the presence of vaccine virus in down feathers of birds vaccinated with different serotypes over time (Islam & Walkden-Brown, 2007). These studies clearly showed the curve progression with the

weekly increase in the percentage of positive birds. In general, the CVI component presented higher replication levels and had already reached its peak plateau at 21 days; for the HVT component it was as late as at 28 days of age (Table 3 and 4, respectively). Specially regarding the CVI component, the difference both in replication rate and in the percentage of positive birds between treatments T01 and T02 is remarkable when compared to T03 which had the lowest result at 21 and 28 days (Figure 1 and Table 3). For proportion of positives, at 14 days, program B had 2.7 times more risk to be positive in CVI than program C ($p < 0.001$). At 21 days, program B had 1.7 times more risk to be positive in CVI than program C ($p = 0.005$). This distinct behavior between different products has also been described in studies of the CVI component (Gimeno et al., 2015a). Comparisons of different vaccination programs using CVI against Marek's disease indicate that each vaccine has unique and specific features that are related to its production process and mainly to the number of passages used in master seed attenuation (Witter et al., 1987; Witter & Kreener, 2004). Thus, each product has unique characteristics which explain the differences in replication rates observed.

Although the replication profile of the HVT component of Marek's vaccine virus was shown to be slower and less intense than the profile of the CVI component, significant differences between treatments could be observed when the HVT component was separately analyzed. Again, in this case, the treatment represented by the conventional combination vaccine (T02) showed a higher replication rate and a higher percentage of positive birds than the treatments using vector vaccines (rHVT), T01 and T03. The difference was statistically relevant between T02 and T01 at 28 days, with T01 showing a lower detection percentage of the rHVT vaccine virus (Table 4). For HVT, at 28 days, program B had 3.2 times more risk to be positive than program A ($p = 0.009$). Results from previously published research on differences between conventional and recombinant HVTs demonstrated that each product behaves differently, and the age of vaccination and dosage also significantly affect vaccine efficacy (Gimeno et al., 2016).

Despite the higher dose administered (see table of titrations of batches used), the average mean level of CVI and HVT DNA load measured by q-PCR was significantly lower in feather tips for vector vaccines (T01 and T03) than for the conventional vaccine (T02). Maternal antibody against infectious bursal disease virus (IBDV) was found in all tested day-old chicks. This antibody would have the ability to neutralize the recombinant rHVT in T01 and T03, but not the conventional HVT vaccine virus in T02 and could be responsible for the lower levels of replication of the recombinant HVT virus. As rHVT expresses antigenic portions of the Gumboro virus on its surface, maternal antibodies could help opsonize the vectorized vaccine by increasing the rate of elimination of the vaccine strain and interfering with the replication of the Marek vaccine.

The effect of maternal immunity in the control of Marek's disease using conventional vaccines is well studied and documented in the literature (Chubb & Churchill, 1969; Eidson et al., 1972), but with the development of vectorized vaccines based on recombinant rHVT, investigations are needed to determine the effect of passive IBD antibodies on rHVT replication rate. However, to definitively answer this, replication of the rHVT in vector vaccines would need to be compared in maternal-antibody-negative, genetically- and age-matched chicks. This was not possible since the commercial parental flocks are always vaccinated.

The fact is that even T02 having Marek's vaccines with lower titers (PFU/dose) than treatments T01 and T03, the replication rate of CVI and HVT components was still higher and faster than in vaccination programs using vector vaccines (T01 and T03). This indicates that the comparison of different vaccination programs should not solely and exclusively consider the titer of vaccines used, since each vaccine has its particular features, and the titer expressed in PFU/dose is not a valid indicator for comparisons between different products. Serial passage through propagation systems (usually in chick embryo fibroblasts; CEF) has long been recognized as a method of attenuating viruses used for vaccine production (Witter et al., 1990). Generally, the more passages the virus undergoes from its original isolation from chickens, the more attenuated (weaker) it becomes. At higher passages, Marek's vaccine causes larger, easier to observe plaques, and since the plaque forming unit (PFU) assay technique requires a count of visible plaques, higher passage vaccines are likely to be given a higher PFU value. However, this high PFU titration may not correlate to their performance in chickens (Witter et al., 1990; Witter & Kreager, 2004). Marek's viruses, which are used to produce Marek's vaccines, can be weakened to the point of non-efficacy by excessive passage levels. Therefore, high passage Marek's vaccines are less effective.

A limitation of this study is the absent of control group without vaccination. Authors consider that in results and applications of them. The results of this study can be used for comparison only between vaccines programs and can not be extrapolated to evidence of effect of vaccines alone in front of non vaccinated animals. Besides this, the results are relevant for that purpose.

CONCLUSIONS

The results shown in this study highlight significant differences between the treatments evaluated. In general, the conventional Marek's vaccine combining CVI and HVT components showed higher replication rate and percentage of vaccine coverage than programs with rHVT vector vaccines. These differences should be considered when poultry producers develop their strategy to control Marek's disease and establish a vaccination program. In addition, the results showed that the associated use of IBD-immune complex vaccines does not impair

replication of CVI and HVT components in Marek's vaccines.

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