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Hatchery Vaccination Quality Control of Herpesvirus of Turkey-Infectious Bursal Disease HVT-IBD Viral Vector Vaccine Application by Specific qPCR

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Abstract: Infectious Bursal Disease Virus (IBDV) control can be achieved using vector turkey Herpesvirus vaccines expressing IBDV immunogenic proteins (HVT-IBD). Vaccination Quality Control (QC) and assurance of a vaccine take are of paramount importance for these vaccines. This work aims to assess the commercial HVT-IBD vector vaccine take and its *in vivo* recovery after hatchery application. A specifically designed quantitative real time polymerase chain reaction (qPCR) assay was used in laboratory reared conventional broilers and from field vaccinates. Results showed positive HVT-IBD vaccine virus PCR detection between 60 and 100% in fresh feathers with a maximum observed at 28 days post-vaccination. Positive samples were consistently shown in fresh spleen tissues and bursas and in fresh Peripheral Blood Mononuclear Cells (PBMC) from day 11. Free range vaccinates were 98% positive at 35 days and 88% at 81 days of age. Overall, the results suggest that the HVT-IBD vector vaccine immunization success was associated to consistent vaccine virus recovery during several weeks post-vaccination. Considering that immunization success in a flock is linked to efficient vaccine virus recovery in a maximized proportion of birds, a tool to monitor this criterion was needed. A qPCR test designed to be specific for the HVT-IBD vector vaccine evaluated in this work was tested with success. The most appropriate samples for vaccination monitoring, fresh feather tips, spleen and PBMC were defined. They allowed studying the kinetics of *in vivo* recovery of the HVT-IBD vaccine. These results contributed to HVT-IBD vector vaccine vaccination QC assessment.

Key words: Hatchery vaccination, quality control, Infectious bursal disease virus, vector vaccine, HVT-IBD, qPCR

INTRODUCTION

Marek's Disease (MD) is an oncogenic and immunosuppressive disease caused by an avian Herpesvirus that has hampered the poultry industry during the past century (Witter, 1982). Infectious Bursal Disease (IBD) caused by the IBDV is immunosuppressive with high morbidity and potential to induce elevated mortality rates (Becht, 1980). IBDV destroys the B lymphocyte precursors present in the bursa of Fabricius inducing atrophy of the organ, mortality and/or immunosuppression in unprotected flocks (Muller *et al*., 2003). These diseases acting alone or interacting with other infectious agents are of foremost importance for the poultry industry that relies on proper husbandry and vaccination for their control (Hoerr, 2010). MD control by vaccination in the broiler industry is successfully achieved by using commercially available vaccines, Turkey Herpesvirus (HVT), SB1 and Rispens (Witter, 1982). On the other hand, IBD control in broilers is attempted using live modified and/or killed vaccines for the breeders and progeny with variable success due to low cross-protection levels and maternally-derived antibody (MDA) interference with immunization among other factors (Rosales *et al*., 1989). *In ovo* or day-old subcutaneous vaccination with HVT vectors expressing the IBDV VP2 (HVT-IBD) allow protection against both MD and IBD (variant and classic) in the presence of high MDA and represent the current trend in the poultry industry (Bublot *et al*., 2007; Goutebroze *et al*., 2003; Perozo *et al*., 2009). However, such HVT-based vaccines show no horizontal transmission and therefore no contact 'vaccinations' after the initial injection, as contrary to live modified IBD vaccines (Purchase and Okazaki, 1971). Verification of sufficient individual vaccine take within the vaccinated population is thus crucial when using such vaccines.

Vaccination Quality Control (QC) can be implemented at the immunization process at different levels by checking for compliance with good vaccination practices such as: vaccine preparation and cold chain consistency to limit

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the loss of titers, application procedures (number of wet birds displaying liquid vaccine on the neck region and speed for subcutaneous administration). After application, vaccination QC can be achieved analyzing the vaccine response (serology), the protection provided (challenge study) and homogeneity of vaccination (percentage of vaccinated animals). A novel method of monitoring the quality of immunization that can be used in the field is the quantitative polymerase chain reaction (qPCR) assay to search for the viral vector DNA (Handberg *et al*., 2001) post-vaccination. MD virus pathogenicity (including HVT) comprises a final stage of replication in the feather follicle, therefore, the virus can be easily re-isolated and detected by molecular techniques from the pulp in the feather tip (Cortes *et al*., 2009). In order to evaluate a non invasive diagnostic tool for the assurance of the vector HVT-IBD vaccine take feather tips was the logical choice for this work. Conditions for importation of infectious agents by the U.S. Department of Agriculture require that these organisms must be inactivated by chemicals, such as phenol or formalin, before being transported (Snyder, 2002). An alternative and safe way of transportation of inactivated microorganisms is represented by the Flinders Technology Associates filter paper (FTA®) (FTA is a registered trademark of Whatman Plc, Maidstone, Kent, UK). The FTA cards are chemically treated filter paper designed for the collection and room temperature storage of biological samples for subsequent analysis (Moscoso *et al*., 2005; Moscoso *et al*., 2004; Natarajan *et al*., 2000; Rogers and Burgoyne, 2000). The FTA cards have been used for multiple molecular studies such as DNA processing from human or wildlife samples (Raina and Dogra, 2002; Rosales *et al*., 1989) and have become a very interesting approach for the detection of poultry microorganisms such as *Mycoplasma* and infectious bronchitis virus and more recently MD virus (Cortes *et al*., 2011; Moscoso *et al*., 2004; Muller *et al*., 2003). Therefore, the vector HVT-IBD vaccine viral genome detection from inactivated DNA on filter paper was preliminary evaluated but did not in the described experimental conditions allow reliable recovery by qPCR. This work aims at evaluating the vaccine take after hatchery application of a commercial HVT-IBD vaccine by using a specific qPCR analysis of fresh tissue. This would help enhancing QC of vaccination protocols using vector vaccines.

MATERIALS AND METHODS

Vaccines: The virus used for vaccination was a vector HVT vaccine virus (strain FC126) whose genome contains the VP2 gene of the 52/70 Faragher strain of IBDV (Goutebroze *et al*., 2003). The resulting strain vHVT013-69 is commercially known as VAXXITEK® HVT+IBD (Merial S.A.S., Lyon, France) (VAXXITEK is a registered trademark of Merial). A batch of HVT-IBD vector vaccine was used to vaccinate the birds manually at one day of age for the laboratory studies. Vaccination of the field study groups was done using a vaccinator machine under hatchery conditions. The dilution and dose were based on the manufacturer's recommendation; each vaccine dose had a volume of 0.2 mL.

Molecular detection of vHVT-IBDV (qPCR assay): A specific vHVT13-069 virus qPCR assay using a beacon probe was developed in house. The probe corresponds to 243 base pairs (bp) of the HVT-IBD genetic construct nucleic acid located between the linker and the VP2 insert (Merial S.A.S., unpublished) and is therefore specific for this HVT-IBD commercially available vector vaccine (sequences of the primers and the probes are available upon request and under contract with Merial S.A.S.). A fluorescent probe specific for a segment between the two primers of the target sequence is cleaved during each amplification cycle, releasing the fluorescent molecule. The fluorescence is then measured as optical density by a spectrophotometer. A validation of the qPCR technique aimed at defining the limit of quantification. A validation of the qPCR technique aimed at defining the limit of quantification. Three identical experiments were performed using two different equipments, A and B. Eight replicates of each pEL098 plasmid dilution (from 10e7 to 10e1 c μL^{-1}) and four replicates of each positive control (T+) dilution (T+ 10e-1 to T+ 10e-5) were tested (Table 1). pEL098 and T+ were diluted in negative control (T-) 10e-1. Samples with Ct values inferior to 38 can all be securely considered as positive according to the limit of quantification studies.

Three experiments, trials I, II and III, were performed in order to validate the qPCR technique *in vivo*. For trials I and III the test was performed on two feathers from the edge of the wing torn from each animal. A total of 50 ng of genetic material (DNA from equivalent to approximately 104 cells) was extracted with the DNEASY® (Qiagen, Valencia, CA, USA) (DNEASY is a registered trademark of Qiagen, Inc.) tissue kit, following manufacturer's instructions. For trial II, DNA extraction was performed on fresh blood, PBMC or tissues (spleen and bursa). Amplification was performed using a duplex format. Thus, vHVT13-069 and beta actin were simultaneously amplified and detected in order to control the efficiency of DNA extraction and amplification and the absence of inhibitors in the samples. Results were expressed by number of cycles or Ct (Cycle threshold). Ct arithmetic means per series of samples were calculated.

Experiment I: From a total of 40 1-day-old commercial broilers, 20 were vaccinated by the subcutaneous route with 0.2 mL of vaccine containing at least 3000 plaque

Table 1: Limit of quantification of the specific to the vHVT013 vaccine virus strain qPCR test in duplex

	Ct mean on equipment 1				Ct mean on equipment 2				S.D. between two equipments
	Run 1	Run 2	Run 3	S.D.	Run 1	Run 2	Run 3	S.D.	
pEL098 10e7 c/µl	14.82	14.66	14.86	0.11	15.33	14.92	14.52	0.41	0.28
pEL098 10e6 c/µl	17.34	17.45	17.82	0.25	18.02	17.86	17.88	0.09	0.27
pEL098 10e5 c/µl	20.94	20.79	21.04	0.13	21.58	21.32	21.17	0.21	0.28
pEL098 10e4 c/µl	23.86	23.64	23.36	0.25	24.81	23.82	23.93	0.54	0.49
pEL098 10e3 c/µl	27.37	27.18	27.33	0.10	27.99	27.60	27.70	0.20	0.29
pEL098 10e2 c/µl	30.71	30.37	30.41	0.19	31.07	30.45	30.71	0.31	0.27
pEL098 10e1 c/µl	34.85	35.35	33.48	0.94	34.73	34.23	33.82	0.46	0.66
H_2O plasmid	0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T+ 10e-1	23.02	22.95	23.90	0.53	24.83	24.30	24.39	0.28	0.77
T+ 10e-2	26.42	26.42	26.60	0.10	27.47	27.05	26.88	0.30	0.41
T+ 10e-3	29.89	29.89	29.66	0.13	30.62	30.17	30.06	0.30	0.33
T+ 10e-4	33.16	33.48	32.94	0.27	34.06	33.28	33.17	0.49	0.39
T+ 10e-5	39.07	39.09	38.79	0.17	40.33	38.82	38.75	0.89	0.60
T- 10e-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_2O sample	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Efficacy	101.9%	98.1%	109.2%	-	103.9%	105.7%	104.6%	-	-

S.D. = Standard Deviation

forming units of the HVT-IBD vector vaccine. The remaining birds were kept as unvaccinated controls; both groups were housed in isolation units where water and feed was provided *ad libitum*. Additionally, 30 commercial birds were vaccinated the same way at day-old in the hatchery, identified with wing bands and sent to be reared under field conditions. At sampling, two wing feathers were collected from each bird and then kept stored in tightly sealed plastic bags. The feathers were kept at room temperature for transportation and stored in a refrigerator (+4°C) until processing. Individual qPCR analysis of each sample for the vaccinated and unvaccinated groups were performed. Feathers from birds in laboratory studies were collected on D20, D28, D35 and D42. For the field reared chickens, feathers were collected fresh from 15 birds on D21 and D28.

Experiment II: A total of 30 'cou nu gris' chicks were obtained from a flock at the 'Centre de Sélection de la Volaille de Bresse' in France while reared under field conditions. All the birds were vaccinated with the HVT-IBD vaccine in the hatchery at one day of age via the subcutaneous route. On days 4, 8, 11, 15 and 18 post vaccination five birds were humanely euthanized each day, then sampled in laboratory conditions to avoid cross-contamination of sampling material. Sampling was performed in birds chosen at random in the flock then individually identified. Blood was collected in 10 mL tubes containing an anticoagulant. It was slowly inverted during 5 to 10 min to obtain a homogeneous mix. 2 mL of blood were kept for blood PCR in a tube. The other part of blood was used for PBMC isolation in a Ficoll gradient by centrifugation during 25 min. PBMC was mixed with 10 mL of suspension containing 2% of foetal calf serum. The suspension was then centrifuged during 5 min. Spleens and bursas were collected in sterile Petri dishes. DNA extraction and qPCR assay were implemented as previously explained.

Experiment III: In order to evaluate vaccine virus take and persistence, coloured long-living broilers from two commercial farms in France (reared as free range after six weeks of age) were studied. Feather samples were obtained from individually identified birds (26 vaccinated and 13 unvaccinated sentinels) at 35, 42, 56 and 81 days from each of five houses included in the study, for a total of 130 samples per sampling day. Feathers were processed and tested for the presence of the HVT-IBD vector vaccine by qPCR as previously explained. Serological monitoring using the IBD ELISA test from Synbiotics Corp. (Kansas City, MO) was performed in the birds at day-old in order to determine the levels of IBD maternally-derived antibodies. It was performed in parallel at the same ages (D35, D42, D56 and D81) in the same identified birds following the manufacturer's instructions. Correspondence between IBD ELISA and qPCR positive birds was studied at the four ages for the vaccinates and at D35 for the sentinel birds, remained unvaccinated.

Time point series of results for the three experiments as described below were compared using non-parametric tests (experiment I laboratory: D20, D28, D35 and D42; experiment I field: D21 and D28; experiment II: D11, D15 and D18; experiment III: D35, D42, D56 and D81). Alpha risk was set up at 5%.

RESULTS AND DISCUSSION

Internal control results demonstrated the validity of the DNA extraction procedure and its standardization. Unvaccinated control bird results showed that no false positive results were found. Experiment I test results are displayed in Table 2, showing that none of the

Table 2: Experiment I: qPCR vaccine virus recovery from day-old vaccinated broilers reared in laboratory conditions and in field conditions

	Unvaccinated (%)				Vaccinated (reared in laboratory conditions) (%)				Vaccinated (reared in field conditions) (%)	
qPCR vHVT-IBD virus detection	D20	D28	D35	D42	D20	D28	D35	D42	D21	D28
Positive vHVT-IBD virus samples	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	6/10 (60)	10/10 (100)	8/10 (80)	9/10 (90)	13/15 (87)	12/14 (86)
Mean Ct of positive birds	-	-	-	-	39.4±1.3	33.0±5.1	35.2±5.4	34.6±5.8	31.7±3.5	31.0±5.6

Table 3: Experiment II: qPCR vaccine virus recovery from day-old vaccinated coloured birds reared in field conditions – early sampling

	Blood samples		PMBC		Bursa		Spleen	
qPCR detection	Positive (%)	Ct*	Positive (%)	Ct*	Positive (%)	Ct*	Positive (%)	Ct*
D4	0/5 (0%)	-	0/5 (0%)	-	0/5 (0%)	-	0/5 (0%)	-
D8	0/5 (0%)	-	1/5 (20%)	35.0±0.0	2/5 (40%)	36.9±0.0	1/5 (20%)	37.2±0.0
D11	0/5 (0%)	-	2/5 (40%)	36.6±3.2	4/5 (80%)	34.1±3.7	5/5 (100%)	34.8±2.8
D15	0/5 (0%)		4/5 (80%)	35.4±1.0	4/5 (80%)	34.3±2.7	5/5 (100%)	32.7±5.1
D18	0/5 (0%)	-	5/5 (100%)	35.7±1.6	5/5 (100%)	34.2±2.8	5/5 (100%)	31.4±2.7

*Mean Ct on positive samples; PBMC = Peripheral blood mononuclear cells

unvaccinated control birds were positive during the study. 60% of the vaccinated birds were tested positive at the first sampling on D20. Gene copy levels were even higher on D28 and 100% of the samples were tested positive. Most of the birds remained positive on D35 (80%) and on D42 (90%). As shown in Table 2, a high inter-individual variability in the Ct values was observed depending on the post-vaccination sampling date. The maximum HVT-IBD vector vaccine expression was observed in feather tip samples around D28 post-vaccination. Nevertheless, series of results did not display a significant difference between dates (D20, D28, D35 and D42). For the field birds tested by direct qPCR on DNA extracted from the fresh feathers test results were comparable. On D21 and D28 86-87% of samples were found positive. Series of results did not display a significant difference between dates (D21 and D28).

The experiment II results are displayed in Table 3. Throughout the study in a total of 192 qPCR analyses, mean Ct values per series were comprised between 40.5 and 31.4. Whole blood samples processed from fresh samples showed that almost no amplification was detected. Analyses using fresh PBMC showed that amplification was possible from fresh samples beginning on D11 and the trend was to obtain increased level of amplification later, after D15. Analysis on bursa of Fabricius samples showed the same trend for increased levels of amplification from D11 up to D18. Spleen analyses displayed a different pattern; amplification was hardly detected from fresh tissues from D8 then increasing to reach the maximum on D11. Series of results did not display a significant difference between dates (D11, D15 and D18). Results for the experiment III in which the HVT-IBD vector vaccine take up to 81 days was tested are shown in Table 4. A high of 98% of the birds were positive for the vaccine genome at D35, the percentage of positive birds slightly decreased with time with 92% on D42, 88% at D56 and up to day 81 still 89% of the birds tested positive to the vaccine. Overall, 92% of the samples tested in this trial were positive (477 /520). Series of results displayed a significant difference between dates ($p = 0.000$) (D35, D42, D56 and D81). Correspondence between qPCR positive results (98%) and IBD ELISA positive results (100%) at D35 of age was established before IBD virus challenge usually detected in field conditions by serology; that was confirmed by the very low percentage of IBD ELISA positive results in the sentinels (9%) (Table 5).

It is essential to remember that in vector vaccine technology no revaccination is recommended and the vector vaccine does not transmit horizontally (Purchase and Okazaki, 1971), indicating that vaccine preparation and vaccination QC is of foremost importance. Monitoring immunization and assuring positive vaccine

Table 4: Experiment III: qPCR vaccine virus recovery from day-old vaccinated coloured birds reared in field conditions-long term experiment

	D35		D42		D56		D81		All dates
qPCR detection	Positive (%)	Mean Ct on positives	Positive (%)	Mean Ct on positives	Positives (%)	Mean Ct on positive	Positive (%)	Mean Ct on positives	Positive (%)
House B1	26/26 (100%)	31.9±0.9	26/26 (100%)	32.4±1.1	23/26 (88%)	32.3±2.5	25/26 (96%)	36.5±1.3	100/104(96%)
House B2	26/26 (100%)	30.9±2.0	26/26 (100%)	33.4±0.4	26/26 (100%)	32.5±2.2	20/26 (77%)	33.4±2.9	98/104 (94%)
House B3	26/26 (100%)	32.8±2.0	26/26 (100%)	32.6±1.9	24/26 (92%)	34.1±1.5	22/26 (85%)	36.1±1.2	98/104 (94%)
House B4	25/26 (96%)	31.7±2.4	18/26 (69%)	36.9±1.1	23/26 (88%)	38.3±0.5	24/26 (92%)	38.4±0.7	90/104 (86%)
House B5	24/26 (92%)	32.6±1.2	23/26 (88%)	34.4±1.3	19/26 (73%)	33.9±1.8	25/26 (96%)	36.0±0.5	91/104 (87%)
Total +ve birds (%)	127/130 (98%)		119/130 (92%)		115/130 (88%)		116/130 (89%)		477/520 (92%)

virus recovery in the bird is the only way to provide protection against IBD and MD. HVT virus accumulates in the epithelial cells of feather follicles from the 10th day after infection and throughout the life of the animal (productive phase of replication) and the amount of viral DNA is 1000 times greater than in other organs (Baigent *et al*., 2005a). In addition, the amount of virus in the feather follicles is correlated with viral load in lymphoid organs (Islam and Walken-Brown, 2007) and therefore antigenic stimulation, making it a good indicator of vaccine protection (Baigent *et al*., 2005b). These feather follicles represent an excellent source of vector DNA, allowing non-invasive sampling and successive testing on the same bird and are the best possible source to monitor the kinetic of the vector (Baigent *et al*., 2005b; Cortes *et al*., 2009). During experiments I and III, the technique of sampling two feathers per bird was tested and allowed obtaining interpretable results; similar procedures were described by Cortes *et al*. (2011). They compared the suitability of blood and feather pulp samples for MD diagnostics and vaccine kinetics monitoring. Their results showed that the load of serotypes 1, 2 and 3 DNA was higher in feather pulp than in blood and reported that diagnosis of MD could be done using both feathers and blood samples but monitoring of MD vaccination by real time-PCR required the use of feather pulp samples. There was a high percentage of false negative samples when using blood to detect serotypes 2 and 3 MDV by real time-PCR. Some lessons learned are that the feathers must be kept as fresh as possible for DNA extraction from the pulp. It is essential to follow the instructions for storage and shipment. The shipment of samples must be done at about +4°C; then feather samples can be stored up to one week at refrigerator temperature. If samples have to be kept more than one week before processing, they should be stored at -20°C. Each sample of two feathers should immediately be put directly into a plastic bag and sealed to avoid cross-contamination between them. The objective of the second trial was to determine the earliest date for sample collection from lymphocyte rich tissues (targets cells for the HVT-IBD vector vaccine virus). As shown by the lack of vector vaccine amplification, whole blood sampling is not a method of choice. These results are in agreement with other researchers who disregarded the use of blood for MD vaccine monitoring (Cortes *et al*., 2009). Based on the detection levels PBMC samples aimed at concentrating the white blood cells can be used if fresh preparations are processed. Sampling is not recommended before D15 to ensure vaccine virus recovery. Bursa sampling may be used if coming from fresh tissues collected after D11. Spleen samples are the most relevant material to gather, from D11 for fresh tissues. An early signal of detection was shown on D8 but in only 1/5 samples, so it is recommended to sample starting on D11.

In vivo replication of the HVT-IBD vector vaccine is very important since long living birds can also be vaccinated with the vector vaccine. This work reports the detection of up to 89% positive birds after several weeks of age, meaning feather pulp samples showed vaccine detection. The biological effect following HVT-IBD vaccine application is sero-conversion detected by IBD ELISA (Lemiere *et al*., 2011). Throughout the experiment III lasting 81 days in field conditions, 100% of the vaccinates were sero-positive, when only 38% of the sentinels were sero-positive. This may be explained by post-IBD challenge sero-conversion. In parallel, overall result 92% of positive using the qPCR technique throughout the study can be correlated to limitations due to the technique, as well as probable vaccine virus *in vivo* reactivations. Significant differences in virus recovery loads throughout the long observation period may be explained this way. In opposite, in a short period of observation around the peak of detection of the vaccine virus, as in experiments I or II, such variability in qPCR results could not be shown in terms of timeline evolution.These overall results are consistent with recent work demonstrating the suitability of HVT-IBD vector vaccines for long lived commercial layers and broiler breeders, inducing clinical protection against IBD and Marek's Disease when associated to a serotype 1 Rispens type vaccine (Lemiere *et al*., 2011).

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REFERENCES

Baigent, S.J., L.J. Petherbridge, K. Howes, L.P. Smith, R.J.W. Currie and V.K. Nair, 2005a. Absolute quantification of Marek's disease virus genome copy number in chicken feather and lymphocyte samples using real-time PCR. J. Virol. Methods, 123: 53-64.

Baigent, S.J., L.P. Smith, R.J.W. Currie and V.K. Nair, 2005b. Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol., 86: 2989-2998.

Becht, H., 1980. Infectious bursal disease virus. Curr. Top. Microbiol. Immunol., 90: 107-121.

Bublot, M., N. Pritchard, F.X. Le-Gros and S. Goutebroze, 2007. Use of a vectored vaccine against infectious bursal disease of chickens in the face of high-titred maternally derived antibody. J. Comp. Pathol., 137Sup1: S81-84.

Cortes, A.L., E.R. Montiel, S. Lemiere and I.M. Gimeno, 2011. Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. Avian Dis., 55: 302-310.

Cortes, A.L., E.R. Montiel and I.M. Gimeno, 2009. Validation of Marek's disease diagnosis and monitoring of Marek's disease vaccines from samples collected in FTA cards. Avian Dis., 53: 510-516.

Goutebroze, S., M. Curet, M.L. Jay, C. Roux and F.X. Le Gros, 2003. Efficacy of a recombinant vaccine HVT-VP2 against Gumboro disease in the presence of maternal antibodies. Br. Poult. Sci. 44: 824-825.

Handberg, K.J., O.L. Nielsen and P.H. Jorgensen, 2001. The use of serotype 1- and serotype 3-specific polymerase chain reaction for the detection of Marek's disease virus in chickens. Avian Pathol., 30: 243-249.

Hoerr, F., 2010. Clinical Aspects of Immunosuppression in Poultry. Avian Dis., 54: 2-15.

Islam, A. and S.W. Walkden-Brown, 2007. Quantitative profiling of the shedding rate of the three Marek's disease virus (MDV) serotypes reveals that challenge with virulent MDV markedly increases shedding of vaccinal viruses. J. Gen. Virol., 88: 2121-2128.

Lemiere, S., S.Y. Wong, A.L. Saint-Gerand, S. Goutebroze and F.X. Le-Gros, 2011. Compatibility of Turkey Herpesvirus–Infectious Bursal Disease Vector Vaccine with Marek's Disease Rispens Vaccine Injected into Day-Old Pullets. Avian Dis., 55: 113-118.

Moscoso, H., O. Raybon, S.G. Thayer and C.L. Hofacre., 2005. Molecular detection and serotyping of infectious bronchitis virus from FTA filter paper. Avian Dis., 49: 24-29.

Moscoso, H., S.G. Thayer, C.L. Hofacre and S.H. Kleven, 2004. Inactivation, storage and PCR detection of mycoplasma on FTA filter paper. Avian Dis., 48: 841-850.

Muller, H., M.R. Islam and R. Raue, 2003. Research on infectious bursal disease-the past, the present and the future. Vet. Microbiol., 97: 153-165.

Natarajan, P., T. Trinh, L. Mertz, M. Goldsboroug and D. Fox, 2000. Paper-based archiving of mammalian and plant samples for RNA analysis. Biotechniques. 29: 1328-1333.

Perozo, F., P. Villegas, R. Fernandez, J. Cruz and N. Pritchard, 2009. Efficacy of single dose recombinant HVT-IBDV vaccination against a variant IBDV strain. Avian Dis., 53: 624-628.

Purchase, H.G. and W. Okazaki, 1971. Effect of Vaccination with Herpesvirus of Turkeys (HVT) on Horizontal Spread of Marek's Disease. Avian Dis., 15: 391-397.

Raina, A. and T.D. Dogra, 2002. Application of DNA fingerprinting in medicolegal practice. J. Indian Med. Assoc., 100: 688-694.

Rogers, C. and L. Burgoyne, 2000. Reverse transcription of an RNA genome from databasing paper (FTA®). Biotech. Appl. Biochem., 31: 219-224.

Rosales, A.G., P. Villegas, P. D. Lukert, O. J. Fletcher, M. A. Mohamed and J. Brown, 1989. Isolation, identification and pathogenicity of two field strains of infectious bursal disease virus. Avian Dis., 33: 35-41.

Snyder, J., 2002. Packaging and shipping of infectious substances. Clin. Microbiol. News., 12: 89-92.

Witter, R.,1982. Protection by attenuated and polyvalent vaccines against highly virulent strains of Marek's disease virus; Avian Pathol., 11: 49-62.