INVESTIGATIONS ON AVIAN ADENO-ASSOCIATED VIRUS BASED PROTEIN EXPRESSION FOR POULTRY VACCINATION, THE VG/GA STRAIN OF NEWCASTLE DISEASE VIRUS (NDV) AND THE USE OF FTA® CARDS FOR NDV DETECTION

by

Francisco Antonio Perozo-Marin

(Under the direction of Pedro Villegas)

ABSTRACT

The avian adeno-associated virus (AAAV) is a replication defective non-pathogenic virus proved useful as a viral vector for gene delivery. The first set of studies aimed the development recombinant AAAV virions expressing the hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) or the viral protein 2 (VP2) of infectious bursal disease virus (IBDV). Transgene expression of the viral proteins was confirmed and the recombinant viruses tested for immunogenicity and protection. The birds vaccinated with the recombinant virions showed IBDV and NDV specific antibodies and up to 80% protection against virulent challenge. The second set of studies aimed to characterize the Villegas-Glisson / University of Georgia (VG/GA) strain of NDV. The VG/GA strain was detected in the respiratory and intestinal tract of chickens with a preferential tropism for the latter. The VG/GA strain and the LaSota strain (used for comparison) induced NDV specific immunoglobulin A (IgA) at the upper respiratory tract. The IgA levels in the trachea were higher for LaSota strain, while in the bile and intestine were higher for the VG/GA strain. Early vaccination with the VG/GA strain afforded 95 to 100%

protection against lethal challenge, equivalent to the protection conferred by LaSota strain. Full genome sequence analysis classified the VG/GA strain within class II, genotype II viruses, which include most of the classic vaccine strains. The third study investigated the feasibility of using Flinders Technology Associates filter paper (FTA® cards) to collect allantoic fluid (AF) and chicken tissue samples for NDV molecular detection. The FTA® cards allowed NDV identification from AF with titter of $10^{5.8}$ ELD_{50}/ml and tissue imprints from experimentally infected birds. The FTA® cards are suitable for collecting and transporting NDV positive samples, providing a reliable source of RNA for molecular characterization and a hazard free sample.

Index words: Newcastle disease virus, infectious bursal disease, avian adeno-associated virus, hemaglutinin-neuraminidase, viral protein 2, VG/GA strain, FTA® card

INVESTIGATIONS ON AVIAN ADENO-ASSOCIATED VIRUS BASED PROTEIN EXPRESSION FOR POULTRY VACCINATION, THE VG/GA STRAIN OF NEWCASTLE DISEASE VIRUS (NDV) AND THE USE OF FTA® CARDS FOR NDV DETECTION

by

FRANCISCO ANTONIO PEROZO-MARIN

DVM., The University of Zulia, Venezuela. 1994.
M.S., The University of Zulia, Venezuela. 2003.

A Disertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR IN PHILOSOPHY

ATHENS, GEORGIA

2008

INVESTIGATIONS ON AVIAN ADENO-ASSOCIATED VIRUS BASED PROTEIN EXPRESSION FOR POULTRY VACCINATION, THE VG/GA STRAIN OF NEWCASTLE DISEASE VIRUS (NDV) AND THE USE OF FTA® CARDS FOR NDV DETECTION

by

FRANCISCO ANTONIO PEROZO-MARIN

Major Professor: Pedro Villegas

Committee: Mark Jackwood
Maricarmen Garcia
Corrie Brown
Stephen Thayer

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2008

DEDICATION

This work is dedicated to my dear wife Yaneth and my three sons Pablo, Francisco and Cesar; (you are worth all the efforts), also to my brothers Luis and Natalio and to my parents Luis and Ana.

ACKNOWLEDGEMENTS

I would like to thank Dr. Pedro Villegas for all his support and kind consideration and for giving me all these opportunities. It has been an honor being his student and becoming his friend during these four years. I hope I will be able to stand to the challenges and expectations in the future based on all what I learned.

I want to acknowledge to my friend Linda Purvis for all her help and patience. We grew together as friends and scientist during these years. I hope for the best in the new life that opens in front of us.

Special thanks to Dr. Carlos Estevez for all his time and dedication in making me enjoy virology research.

Thanks to my new people: Luciana, Ivan, Angela, Alejandro, Ivomar, Andres, Taylor, JJ, etc…. for all of your friendships and for all the fun that we had and will keep having.

Thanks Dr. Rafael Fernandez in Merial for his friendship and support.

Thanks to all the staff and faculty at PDRC for your advice, help, and support.

Thanks to the Universidad del Zulia for the financial and institutional support to my entire career.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Part 1. Purpose of this study

Newcastle Disease (ND) and Infectious bursal disease (IBD) are worldwide distributed, highly contagious diseases of young chickens with high economic significance to the poultry industry. Vaccination of commercially reared birds is the best way to reduce losses resulting from Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) infections. Live or inactivated vaccines currently used for the control of these diseases are derived from attenuated field strains. Live attenuated vaccines are infectious and the inactivated vaccines may result in disease emergence due to lack of antigenic stimulation at the natural site of virus entry. To avoid some of these limitations, the poultry industry is considering the use of recombinant viruses for infectious diseases control. The use of recombinant viruses for gene delivery as a vaccination strategy in veterinary medicine is an ongoing trend derived from advances in the understanding of viral diseases pathogenesis and of the molecular mechanisms involved in the generation of protective immune responses.

The adeno-associated virus (AAV) is a replication defective virus member of the family *Parvoviridae* successfully used for gene delivery in humans and other species. These replication defective parvoviruses are non-pathogenic, capable of accommodating relatively long pieces of DNA and of infecting a wide variety of cell types. The avian adeno-associated virus (AAAV) has

been used for reporter gene delivery in chicken embryo cells. The aim of these studies was the generation of recombinant AAAV virions expressing the hemagglutinin neuraminidase HN protein of LaSota strain of NDV and the viral protein 2 (VP2) of the Edgar strain of IBDV to assess their ability to generate protective immunity in chickens.

The Villegas-Glisson / University of Georgia (VG/GA) strain of NDV have been proposed to replicate both in the respiratory and intestinal tract, with preference for the intestine. It has been established that the mucosal immunity represented by immunoglobulin A (IgA) production play an important role in the development of protection in chickens vaccinated against ND. Antibody production in the mucosa is closely related to viral replication in the target cells; hence the pathogenesis and tissue tropism of the viruses used for vaccination is to be considered in order to assess the efficacy of a given live vaccine against a direct challenge.

The strain intestinal tropism of VG/GA and the consequent induction of local immunity may be important for protection against velogenic-viscerotropic strains of NDV. The VG/GA strain, when applied to immune competent specific pathogen free (SPF) chickens, induces protection against lethal NDV challenge. Peer reviewed reports on the protection conferred to maternal antibody positive broilers is lacking. In this study, virus distribution, local and systemic humoral immune response in SPF birds and the protection against lethal challenge conferred by vaccination with the VG/GA strain in commercial broiler chickens were evaluated and compared with LaSota strain of NDV. In addition, complete nucleotide sequencing and full genome analysis of the VG/GA strain were performed to assess the genomic basis of the strain phenotype.

Conditions for importation of infectious agents by the U.S. Department of Agriculture require that infectious agents must be chemically inactivated before being transported. An

alternative and safe way of transportation of inactivated microorganisms is represented by the Flinders Technology Associates filter paper (FTA® card) that is a chemically treated filter paper designed for the collection and room temperature storage of biological samples for subsequent analysis. A virus inactivation process able to ensure high quality nucleic acids for molecular pathotyping, would be an improvement in field sampling and shipping of NDV for diagnosis means. The FTA® cards have been used for multiple molecular studies such as DNA processing from human or wildlife samples and lately it has become a very interesting approach for the detection of poultry microorganisms; however, no work had been published on its use in NDV nucleic acid detection. The aim of this study was to use the FTA® card as an alternative for the collection and easy transportation of NDV field samples.

Results from these studies are expected to contribute to the understanding of the interaction between recombinant vaccination and the chicken immune response, by demonstrating the feasibility of developing and using an avian adeno-associated virus based gene delivery system for poultry. The results are also expected to assess the unique characteristics of the VG/GA strain of NDV and to improve diagnostic techniques for molecularly identifying NDV with the use of the FTA® cards for nucleic acid transportation.

CHAPTER II

LITERATURE REVIEW

Newcastle disease virus.

- **Generalities**

Newcastle Disease Virus (NDV) is the causative agent of Newcastle disease (ND), a major disease of poultry worldwide. Even though mass immunization is widespread as a management practice, velogenic strains are considered endemic in many countries of Asia, Africa, Central and South America (11, 131). NDV belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, it is one of the members of the genus *Avulavirus*, (76). NDV contains a non-segmented single-stranded RNA genome of negative polarity. The viral RNA encodes six major virus proteins; the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin - neuraminidase (HN) and the large RNA polymerase protein (L) (5, 93). The molecular basis for NDV pathogenicity has been shown to be highly dependent on the amino acid sequence of the F protein cleavage site. Cleavage of the precursor glycoprotein F0 to F1 and F2 by host cell proteases is required for the progeny virus to become infective (93, 145). Dibasic amino acids, surrounding the glutamine residue at position 114 and a phenylalanine in the position 117, are present in mesogenic or velogenic strains, this motif is used as a molecular marker of virulence (74, 93, 145).

Newcastle disease vaccines to control detrimental effects of sub-clinical forms of the disease and extremely expensive outbreaks are commonly used in the poultry industry, which relays on the

use of live and inactivated NDV strains and in the control of immunosuppressive diseases to decrease economic losses (4, 10, 16, 131). Research and development efforts had been targeted to find a reliable and efficient vaccine, able to solve the common drawbacks of the actual vaccination programs, such as the interference with maternal antibodies, strong post-vaccine respiratory reactions and lack of uniformity in vaccine distribution (6). The choice of vaccines should be based on equating the degree of immunity necessary, the costs involved and the exposure to local field viruses, both in terms of their virulence and prevalence. In Latin America and Asia, velogenic strains are considered endemic, so vaccination strategies are targeted to control the virus and to avoid outbreaks (131). For primary NDV chicken vaccination, the vaccine of choice is one that elicits immune response with minimum respiratory reactions (10).

- **Definition**

According to the World Organization of Animal Health (formerly the office of International Epizooties and still recognized by the abbreviation OIE), "Newcastle disease is defined as an infection of birds caused by a virus of avian *paramyxovirus* serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater, or: b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term "multiple basic amino acids" refer to at least three arginines or lysines residues between residues 113 to116. Failure to demonstrate this amino acid residues pattern would require characterization of isolated viruses by the intracerebral pathogenicity index (ICPI) test. In this definition, the amino acid residues are numbered from the

N-terminus of the amino acid sequence deducted from the nucleotide sequence of the F gene, the position 113-116 correspond to the residues -4 to -1 from the cleavage site (104).

- **History**

The first recorded outbreaks of NDV go back to 1926 in Java, Indonesia and Newcastle-Upon-Tyne, England. It was called Newcastle disease (ND) by Doyle to avoid confusion with other diseases like fowl plague, now called highly pathogenic avian influenza (11, 12). Three hypotheses on the emergence of NDV have been postulated: a) the virus has always been present as an unrecognized disease of chickens and changes in the host population resulted in selection and emergence of the virulent virus. b) The virus was endemic in some other species in witch produced no diseases. c) The virus was originated at some point in time and place as the result of a major genomic mutation (10).

- **Etiology**

Virus classification:

Order Mononegavirales

-Family Paramyxoviridae

-Subfamily Paramyxovirinae

-Genus Respirovirus – bovine and human parainfluenza

-Genus Rubulavirus – Mumps virus and other parainfluenza

-Genus Morbillivirus - measles, canine distemper, rinderpest

-Genus Henipavirus – Hendravirus, the equine (Australia) and the more recent Nipahvirus of pigs (Malaysia) – both animal and human pathogens

-Genus Avulavirus (76) - avian paramyxoviruses are classified as nine serotypes (APMV-1 through APMV-9). NDV isolates are members of a single serotype designated APMV-1.

-Subfamily Pneumovirinae

-Genus Pneumovirus – bovine and human respiratory syncytial virus

-Genus Metapneumovirus – avian and turkey rhinotracheitis (TRT) virus.

Infections of birds with strains of avian paramyxovirus serotype 1 (APMV-1), are associated with two clinical outcomes:

1. Lentogenic NDV infections are typically the cause of mild or unapparent clinical forms unless exacerbated by complicating infections.
2. ND results from infections with virulent APMV-1, called Exotic ND in U. S. Code of Federal Regulations, also called virulent NDV (v-NDV) or just velogenic NDV in endemic countries. Virulent forms of the disease are reportable to regulatory agencies OIE and there is an impact in poultry international trade, the strains are considered “select” agents after 9/11 regulations.

Descriptive names are used to differentiate the large variation in clinical forms of the disease in chickens caused by different virus strains that are indistinguishable in serological tests because they are members of a single serotype (8). The five forms (pathotypes) with the strains associated with each form include:

1. **Doyle's form** - viscerotropic velogenic Newcastle disease - acute, lethal with hemorrhagic gut lesions, nervous signs may be seen [(Fontana (CA1083), Herts 33, or CA END 2002 strains)].
2. **Beach's form** (avian pneumoencephalitis) - neurotropic velogenic ND - acute, often lethal, respiratory and neurological signs, but no hemorrhagic lesions –USA 1930s to early 1970s (Texas GB strain).
3. **Beaudette's form** - mesogenic - less pathogenic, deaths only in young birds (Roakin). Isolation of this pathotype from poultry is infrequent except in countries where mesogenic vaccines are used.
4. **Hitchner's form** – a lentogenic pathotype - mild or unapparent respiratory disease - typical of common live vaccines (C2, B1, LaSota, VG-GA).
5. **Asymptomatic** another lentogenic type - predilection for gut over respiratory tissue, typical of the indigenous strains in Australia (Queensland V4) or Ulster strain.

Characterization of the virulence of NDV isolates requires laboratory evaluation because the immune status and susceptibility of different hosts may present a different clinical picture that that observed in susceptible chickens inoculated with the same isolate. The development of standardized tests was critical to the assessment of pathogenicity. The mean death time in embryonated eggs (MDT) and the intracerebral pathogenicity index (ICPI) in day-old chickens

were initially used as a practical method for typing and differentiating low virulence (lentogenic) and moderate virulence (mesogenic) NDV vaccine strains (41). Long times to embryo death (>90 hrs) were correlated with low virulence and low ICPI indices. The determination of an intravenous pathogenicity index (IVPI) in 6-wk-old SPF chickens is used to differentiate the high virulence isolates (velogenic) from the mesogens (7).

In the U. S. the intracloacal inoculation of 6- to 8-wk-old SPF chickens has been used to differentiate between velogenic and neurotropic NDV isolates. Viruses are classified as velogenic if intracloacally inoculated chickens develop clinical signs and die. The presence of hemorrhagic lesions in chickens infected with velogenic viruses differentiates them from those infected with neurotropic viruses. Requirements for conducting the ICPI, IVPI, and intracloacal inoculation tests are NDV susceptible specific-pathogen-free (SPF) chickens and appropriate biocontainment facilities (8).

- **Economic importance.**

The global economic impact of ND is enormous. It is certainly unsurpassed by any other poultry virus and probably represents a bigger drain on the world's economy than any other animal virus. In developed countries with established poultry industries, not only are outbreaks of virulent ND extremely costly, but control measures, including vaccination, represent a continuing loss to the industry (69). Even countries free of virulent ND usually face the cost of repeated testing to maintain that status for the purposes of trade. In many developing countries virulent ND is endemic and therefore, represents an important limiting factor in the development of commercial poultry production and the establishment of trade links (131).

- **Virus characteristics.**

NDV is a single stranded, negative sense unsegmented virus, with pleomorfic mid size virions. Nucleotide sequence of the NDV genome has shown to consist of 15,186 nucleotides. The genome of NDV codes for six proteins, the nucleocapsid protein (NP), the phospoprotein (P), the matrix protein (M) the fusion protein (F) that forms the smaller of the surface projections; the hemagglutinin-neuraminidase (HN) responsible for forming the larger of the two types of projections seen on the surface of paramyxovirus particles and the RNA-directed RNA polymerase associated with the nucleocapsid (L) (93). The order of the genes for these proteins in the virus genome is 3´- N-P-M-F-HN-L - 5´. Two non structural V and W proteins generated by RNA editing from the P gene are recognized (71, 125, 144). The virions are enveloped with a lipid bilayer membrane. Projections of approximately 8 -12 nm are observed in the viral surface, corresponding to the F and HN glycoprotein spikes (8).

- **Replication.**

The strategy for replication employed by NDV is that of the negative strand viruses in general, as detailed by Lamb and Parks, 2007 (66). Briefly, the initial step is attachment of the virus to cell receptors, mediated by the HN polypeptide. Fusion of the viral and cell membranes is brought about by action of the fusion (F) protein, and, thus, the nucleocapsid complex enters the cell. Intracellular virus replication takes place entirely within the cytoplasm. Because the virus RNA has negative sense, the viral RNA-directed RNA-polymerase (transcriptase) must produce complementary transcripts of positive sense that may act as messenger RNA and use the cell's mechanisms, enabling the translation into proteins and virus genomes. The viral proteins synthesized in an infected cell are transported to the cell membrane, which becomes modified by

their incorporation. Following the alignment of the nucleocapsid close to modified regions of the cell membrane, virus particles are budded from the cell surface.

- **Pathology.**

Clinical signs.

Newcastle disease virus isolates can be broadly grouped into pathotypes on the basis of clinical signs, which in turn are affected by the strain of virus. Other factors also important in establishing the severity of the disease are the host species, host age, host immune status, co-infection with other organisms, environmental stress, social stress, route of exposure, and the virus dose (11). With extremely virulent viruses, the disease may appear suddenly, with high mortality occurring in the absence of other clinical signs. In outbreaks in chickens due to the virulent ND, clinical signs often begin with listlessness, increased respiration, and weakness, ending with prostration and death. This type of ND may cause edema around the eyes and head. Green diarrhea is frequently seen in birds that do not die early in infection, and prior to death, muscular tremors, torticollis, paralysis of legs and wings, and opisthotonos may be apparent (12).

The neurotropic velogenic form of ND has been reported mainly in the United States. In chickens, it is marked by sudden onset of severe respiratory disease followed a day or two later by neurological signs. Egg production falls dramatically, but diarrhea is usually absent. Morbidity may reach 100% and mortality is variable reaching up to 50% in adult birds and 90% in young chickens (12). Mesogenic strains of NDV usually cause respiratory disease in field infections. In adult birds, there may be a marked drop in egg production that may last for several weeks. Nervous signs may occur but are not common.

For lentogenic NDV infections, mortality in fowl is usually low, except in very young and susceptible bird, but may be considerably affected by exacerbating conditions. Lentogenic viruses do not usually cause disease in adults. Following infection of young, fully susceptible birds, with the more pathogenic strains such as LaSota, serious respiratory disease problems can be seen often resulting in mortality (8).

Transmission

Virus shed from respiratory and intestinal tract of infected birds is transmitted by aerosol and/or ingestion. The disease associated with fecal/oral transmission appears to spread more slowly through a flock than spread from respiratory infections. The virus spread can occur by direct contact with infected birds including wild birds, contaminated personnel and equipment, contaminated feed or water, infected broken eggs or virus-laden feces on eggs in incubators (10). The incubation period of ND after natural exposure has been reported to vary from 2-15 days (average 5-6 days) (8). The overall clinical consequences vary with strain of virus, dose, route of exposure, immune status, age, and host species, ranging from sub-clinical infection to 100% mortality in a short period of time. Isolates that produce mortality in pigeons may produce only mild signs in chickens and viceversa. Chickens most susceptible species, turkeys more resistant, generally waterfowl are believed to be quite resistant (12).

Gross lesions

Macroscopic manifestations are usually unremarkable except in birds infected with viscerotropic velogenic viruses. Velogenic NDV induced lesions include eyelid edema,

perithymic hemorrhage and occasionally edema, splenic necrosis, and necrohemorrhagic lesions distributed through the intestinal site but localized at the sites of lymphoid aggregation (12).

Histopathology.

Histopathological evaluation of virulent NDV infected birds reveals spleens devoid of mononuclear cells and replaced with fibrin deposits, massive destruction of intestinal lymphoid tissue, and disruption of cardiac myofibers with mononuclear infiltrates. Some birds with VVND also have focal neuronal degeneration and gliosis. The primary lesions of neurotropic velogenic ND are neuronal degeneration and variable disruption of heart muscle. Birds infected with less virulent isolates had splenic hyperplasia, myocardial inflammation with degeneration of some myofibers, and mononuclear infiltrates. (22, 63-65)

Molecular basis of pathogenicity and virulence of NDV

Relatively little is known concerning the contributions of the various viral proteins to virulence. The hemagglutinin-neuraminidase (HN) protein provides the initial virus attachment and the fusion (F) protein functions to fuse virus and cell membranes for entry of the virus nucleocapsid into the cell. These two NDV surface glycoproteins are critical to establish a cellular infection. Currently, the molecular basis of pathogenicity of NDV is determined by the amino acid sequence of the cleavage site of the F protein (88, 104, 117). Nevertheless, artificially created infectious clones of known genetic sequence suggest that the HN (93) and V (50, 78) proteins also play a role in the pathogenicity of NDV isolates.

A molecular basis of the F protein cleavage site motif is based on the fact that the F protein, which functions to fuse virus and cell membranes for entry of the virus nucleocapsid into the cell, is activated upon cleavage by host enzymes. It is synthesized as a precursor, FO, and is only fusogenic after cleavage into disulfide-linked F1 and F2 polypeptides by host proteases (38). Thus, NDV becomes infective only when the precursor glycoprotein FO is cleaved in F1 and F2.

It was noted by Rott et al. in the 1970s [cited by Alexander, 1998 (12)], that viruses classified as lentogens would replicate in some cell cultures only if trypsin was added to the medium, whereas those classified as mesogens and velogens would replicate in the same cells without special additives. The amino acid sequence of viruses of low virulence is cleaved by a protease present in a restricted number of cells whereas the cleavage site in more virulent viruses is cleaved by protease(s) present in a wide range of cells and tissues. Determination of the amino acid sequence of the cleavage site provides an indication of the potential virulence of an isolate (117).

Isolates from pigeons have presented some notable difficulties in interpretation of the standard and molecular pathotyping tests. Pigeon NDV isolates, sometimes identified as pigeon PMV-1, often have MDT greater than 90 hrs, a characteristic of lentogenic viruses and ICPIs greater than 1.0 a property of more virulent NDV strains (62). Some of these viruses show a typical virulent amino acid motif at the F protein cleavage site and are virulent in pigeons but show moderate or low pathogenicity in chickens (25). There is some evidence that viruses from species other than chickens don't demonstrate their true virulence until they have been passaged in chickens (10).

Recent studies have demonstrated an incomplete correlation between the above mentioned motif and NDV virulence (30, 93, 96, 104, 105). In these articles the ICPI values of lentogenic viruses mutated to include a virulent virus motif were less than the fully virulent viruses (93, 96, 105), suggesting that the F cleavage site influences, but is not the only determinant of NDV virulence. The HN protein might also contribute to virulence (144), the importance of the interaction between the two glycoproteins for virus infectivity has been properly established (126). HN chimeras of virulent and avirulent virus altered viral pathogenesis (51, 133).

Diagnosis

Gross lesions of virulent NDV are similar to lesions of highly pathogenic avian influenza and are therefore only suggestive of a possible diagnosis. Therefore, definitive diagnosis requires virus isolation and confirmation that the virus is fulfilling the definition of ND that would distinguish it from vaccine viruses or avirulent NDV (3, 8). Briefly, sampling is accomplished with oropharyngeal and/or cloacal swabs, tissue samples from necropsied birds may be included. Most isolates are readily propagated in embryonated chicken eggs. Detection of hemagglutination in culture fluids and inhibition of that hemagglutination with NDV specific antiserum confirms the diagnosis of an APMV-1 infection (9).

Biological characterization of the virulence of an isolate usually requires chicken inoculation but a presumptive indication of virulence potential may be accomplished by noting the time to death of inoculated embryos (more rapid death usually indicates a more virulent virus) and by determining the deduced amino acid sequence of the fusion protein cleavage site from nucleotide sequence analysis of reverse transcriptase polymerase chain reaction (RT-PCR) product of fusion

gene (3). Increased serological titer in samples from a poultry flock is a presumptive evidence of infection. Confirmatory virus isolation may only be attempted if there is an associated clinical disease (12). Serology is usually done by enzyme linked immunosorbent assay (ELISA) because of the capability of automating multiple assays. Hemagglutination-inhibition (HI) (13), is still used widely and measures virtually the same response as virus neutralization (VN). HI and VN detect antibody responses to only the HN and F surface glycoproteins and therefore provide a better prediction of protection against disease than the ELISA test (127).

NDV Detection and further characterization using molecular techniques, such as reverse transcriptase polymerase chain reaction followed by assessment product by restriction fragment length polymorphism analysis (17, 55, 77) or direct nucleotide sequencing (3, 39, 97, 117), represent a diagnostic tool used worldwide.

Vaccination and immunity.

Newcastle disease vaccines to control detrimental effects of sub-clinical forms of the disease and extremely expensive outbreaks are commonly used in the poultry industry, which mainly relays on the use of live and inactivated lentogenic strains (B1, LaSota, Ulster, QV4 and VG-GA) for ND control programs (8, 11, 131) The live virus vaccines are usually applied by mass application methods, i.e. coarse spray, by aerosol, or in the drinking water. Birds must be individually injected with the inactivated vaccines. These vaccines are protective against disease but not against infection, therefore, the use of vaccines in flocks exposed to virulent challenge can make it more difficult to detect virulent infections.

Research and development efforts had been targeted to find a reliable and efficient vaccine able to solve the common drawbacks of the actual vaccination programs, such as, the interference of maternal antibodies, strong post-vaccine reactions and lack of uniformity in vaccine distribution (12). The choice of vaccines should be based on equating the degree of immunity necessary, the costs involved and the exposure to local field viruses in terms of their virulence and prevalence. In Latin America and Asia, velogenic strains are considered endemic, so vaccination strategies should be targeted to control the virus and to avoid outbreaks (131). For primary NDV chicken vaccination, the vaccine of choice is one that elicits immune response with minimum respiratory reactions (8).

The Villegas-Glisson strain of the University of Georgia (VG/GA) has been recognized as a heat resistant enteric strain, with the ability to replicate in the intestinal tract of birds (95). The VG/GA is able to induce protection in chickens to a contact challenge with velogenic strains of NDV (19, 98). The preference of the virus for intestinal replication is extremely important for protection against velogenic-viscerotropic strains of NDV with tissue tropism for intestinal epithelia, where cellular mediated immunity (CMI) and local antibody production are required (118). As a consequence from the replication pattern of the VG/GA strain it is possible to avoid severe vaccine-induced reactions (91, 98).

The reason why VG/GA and other enteric NDV strains target intestinal epithelia cells is unknown, but it may be due to differences in the hemmaglutinin-neuraminidase (HN) protein, witch recently has been reported as a major determinant of tropism and virulence of the isolates (31, 51). The nucleotide sequence of the cleavage site of the VG/GA fusion protein has been

reported as the same to those of LaSota, and B1 (117). Nevertheless, evident differences in virulence and tropism exists (94). Further research is required to establish the correlation between the conformation of HN and the differential tropism of enteric strains.

Most of the lentogenic vaccines are able to induce antibodies against NDV; nevertheless, systemic humoral immune response measured as the presence of specific NDV antibodies in serum is not enough for protection (102, 103). Mucosal immunity represented by local antibody production and cell mediated immunity (CMI) plays an important role in the development of protection in chickens vaccinated against Newcastle disease (103, 116). One of the first steps for a CMI response after a viral infection is the recognition of infected cells by the natural killer (NK) cells and then by the CD8 T cells (recognize Class I MHC presented peptides) which will induce apoptosis or cytolysis in the target cells (35).

Another important component of the CMI is the antigen presenting cells (APCs) delivery of antigenic peptides to the CD4 T-cells using the Class II MHC molecules (B-L in birds) and other adhesion molecules. After activation, Th cells proliferate and can differentiate into effector cells or memory cells; Th effector cells are involved in both CMI and humoral immune response regulation, through cytokine production and cell surface interaction with the target cells (35). All the previously mentioned CMI mechanisms have been observed in the chickens' immune response against NDV (118).

The CMI and local antibody production are closely related to viral replication in the target cells, so the pathogenesis and the sites of replication of the virus used for vaccination, has to be

considered in order to assess the capability of a given live vaccine to protect the bird against a direct challenge (59). The importance of local antibodies in the defense mechanism against viral infection has been emphasized in the recent years (116). It appears that local immunity is of paramount importance since it acts as a barrier at surfaces where primary viral infections occurs, thereby interfering with further spread of the virus (59, 111).

Protection from re-infection by NDV has been best correlated with prior exposure of the epithelia to the infectious agent and high antibody levels (11). Nevertheless, a frequent observation during vaccine-challenge experiments is the resistance to challenge with virulent strains, exhibited by vaccinated chickens that display low levels or even no detectable HI antibodies (59). This is especially important after vaccination by non-parenteral routes using live vaccines; meaning that protection induced by NDV vaccination is not entirely dependant on the production of systemic antibodies (103).

Birds have a well-developed mucosal immune system; its characteristics include local production and secretion of IgA antibodies and trafficking of IgA producing plasma cells (2, 59, 146). Humoral immunity usually appears within 6-10 days after vaccination not only in the blood but also locally. Mucosal antiviral immunity is believed to depend on locally produced antibodies released across the epithelium onto the mucosal surfaces. Although some of these antibodies, mainly IgG, probably are transudated from the blood (112), while IgM and IgA are mainly produced locally (e.g., in the paraocular tissues) and are actively transported through the epithelial cells (36). The IgA class predominates in most secretions and has been reported to be detectable in tears, saliva, tracheal and intestinal washes and bile (2, 36, 59, 112).

Infectious bursal disease virus.

Infectious Bursal Disease Virus (IBDV)

- **History of IBDV**

Infectious Bursal Disease virus (IBDV) is the etiological agent of Gumboro Disease or infectious bursal disease (IBD). The disease was first described in 1957 by Cosgrove (26). Originally identified as "avian nephrosis", the syndrome quickly became known as "Gumboro disease" (26). This virus is a highly contagious disease of young chickens characterized by lymphoid destruction that occurs in the bursa of Fabricius which commonly produces a severe immunosuppression (106, 107). The clinical features of the disease include whitish or watery diarrhea, anorexia, depression, prostration, trembling and sometimes death. Upon necropsy, birds often exhibit dehydration, hemorrhages in the leg and thigh muscles, urate deposits in the kidneys and depending on the stage of the disease, an enlargement of the bursa (67, 81).

In earlier work, the disease was believed to be caused by a strain of infectious bronchitis virus (IBV) because of the presence of urates in the kidneys and the concurrent infection with IBV that was often found in the field (67). After several studies, Winterfield *et al*. (135, 136) succeeded in isolating the causative agent in embryonating chicken eggs and later Hitchner named the disease "infectious bursal disease" (47, 48).

In the early 1980's, the broiler growing area in the Delmarva peninsula began experiencing a significant increase in mortality, however, the clinical syndrome appeared different than previously seen, often with respiratory signs. Bursal lesions ranged from moderate to severe,

often resulting in death because of an *E. coli* infection. Using sentinel birds, Rosenberger *et al*. (108) isolated four IBD viruses identified as A, D, G, and E. These isolates differed from the standard IBDV strains in gross lesions, and by producing rapid bursal destruction with minimal inflammatory response that was typically seen. The available vaccines did not provide complete protection against these new isolates when challenged with the standard challenge strain (STC). These isolates were therefore designated as antigenic variants and new vaccines were developed, tested in challenge studies and proven effective against these variants. Currently, these field variant viruses can be found throughout the United States (15, 28, 46).

In the late 1980's high mortality outbreaks of the disease were being reported in Europe by Chettle *et al.* and van den Berg *et al*. (23, 129). These outbreaks occurred where standard biosecurity and prophylactic measures had already been taken, indicating a drastic change in the field viruses (122). Although no antigenic drift changes were detected, these viruses were labeled very virulent IBDV (vvIBDV) strains because of the increase in mortality and virulence. These strains have now spread throughout Asia, Europe and Latin America (15, 92, 101).

- **Etiology, viral genome and organization**

IBDV is a member of the *Birnaviridae* family, which includes three genera: *Aquabirnavirus, Avibirnavirus* and *Entomobirnavirus*. Among the most noted features of this virus is the bisegmented nature of the genome, as well as the double-stranded RNA. IBDV particles are non-enveloped with a diameter of approximately 70 nm. The virus is icosahedral in shape with a geometric skew of T = 13 (27, 44, 45, 109) . There are two known serotypes of IBDV. Both serotypes affect chickens and turkeys. Serotype 1 is the pathogenic form of IBDV in

chickens and has several antigenic variations in the field (109). Serotype 2 is considered apathogenic for chickens and does not cause lesions. However, one recent report showed that it could be pathogenic in chicken embryos (1). The genome of IBDV is bisegmented, double-stranded RNA with two segments that can be detected by polyacrylamide gel electrophoresis (79). The two segments are designated segment A and segment B. The molecular weight of the two double stranded segments is 2.2 x 10^6 and 1.9 x 10^6 Da, respectively (80). The segment lengths are 3.2 kb and 2.8 kb, respectively. Segment A contains two partially overlapping open reading frames (ORF). The larger ORF encodes a polyprotein which, through auto processing, matures into VP2, VP3 and VP4 viral proteins. The shorter overlapping ORF encodes the non-structural polypeptide known as VP5.

The VP2 is the structural protein that composes the external surface of the virus, it contains a hypervariable antigenic region responsible for serotype specificity and for producing conformational dependant neutralizing antibodies (18, 33, 124). Because these neutralizing antibodies bind to this region of the virion, it is susceptible to mutations and antigenic shifts and is the basis for molecular determination for serotype I viruses. Most of the changes that occur in the VP2 are located between amino acid 203 and 350 (27). VP3 is also a structural protein and is found only on the inner surfaces of virus-like particles. Recent findings indicate that VP3 may play a key role in assembly as well (27). VP4 is a viral protease involved in protein processing and it has been suggested that VP4 also plays a role in activation of the VP1. VP5 was the final viral protein identified in IBDV. This protein plays a role in viral pathogenesis but is not essential for viral replication *in vitro* or *in vivo* (82, 83).

The smaller genome segment (B) encodes for the VP1, the 95 kDa RNA-dependant RNA polymerase (RdRp) responsible for the replication and synthesis of mRNA. The VP1 shares numerous sequence features with other RNA polymerases from various origins (70, 132).

- **Forms of IBDV**

The classic form of IBDV was described in the early 1960's. This form generally affects birds around 3 weeks of age and has an incubation period that ranges from 2 to 4 days. It is characterized by an acute onset of depression in the birds. Often times the birds are reluctant to move, have ruffled feathers, and a decreased feed intake. Severely affected birds become so depressed they become dehydrated and die (26, 37, 72, 108).

The immunosuppressive or sub-clinical form is caused by low-pathogenicity forms of IBDV and is commonly described and seen throughout the United States (14, 15, 110, 123). These strains are often labeled "variant" strains, such as the Delaware variants or the GLS strains (123). These viruses resist neutralization with antibodies developed against the classic forms of the disease and show molecular characteristics that are different from the classic forms. Although these strains are not considered to be highly pathogenic, they can cause severe immunosuppression which leads to vaccination failures and predisposition to opportunistic secondary infections (106).

The very virulent form of IBDV was first described in Europe and has since spread to almost every continent in the world. This viral form is caused by hypervirulent strains of IBDV and is characterized by an acute progression of the classic form leading to extremely high mortality

rates on affected farms. There have been several publications on the molecular basis of vvIBDV, showing that they are distinctly different molecularly and antigenically from both the classic and the immunosuppressive forms of the disease (23, 92, 129).

- **Diagnosis of IBDV**

Over the past 20 years, diagnostic tools have drastically improved making diagnosing of IBDV much easier than in the early years of the disease. Gross lesions and clinical diagnosis is based on the signs of lesions in the bursa of Fabricius, the target organ for IBDV. The gross lesions range from moderate to severe depending on the pathogenicity of the virus.

Histological diagnosis is commonly used because the lesion in the bursa is very characteristic of the disease. This type of diagnosis is especially useful in determining the pathogenicity of the virus, as well as staging the time of infection. Because the virus depletes B cells present in the bursa, histopatological scores are commonly given to the bursa according to the depletion that is observed. This scoring system ranges from 1 to 4, with 1 being a normal bursa and 4 being a totally lymphocyte depleted bursa (120). However, there are other agents that can cause lymphocytic depletion, so a histopathological evaluation can not be the sole determinant for IBDV.

Serological diagnosis is a commonly used tool in diagnosing IBDV as well as monitoring maternal antibodies for IBDV. Current tests include serum-virus neutralization tests and the enzyme-linked immunoabsorbant assay (ELISA). ELISA is more widely used because it is commercially available, rapid and sensitive (75, 109).

Virus isolation was one of the first tools used for diagnosis of IBDV (109). The virus can be isolated and propagated in embryonated chicken eggs, cell culture or in susceptible birds. Propagation in susceptible birds is preferred to the other methods because other methods may cause mutations to the original virus (109).

Viral antigens may be detected as a mean of confirming the presence of IBDV. There are several different methods of detection, including direct or immunoflourescence, immunohistochemistry, agar gel immunodiffusion and antigen-capture ELISA (AC-ELISA) (40, 49). Monoclonal antibodies against IBDV were used for many years as a way to differentiate between strains identifying specific epitopes on the virus capsid (121, 122, 130).

Over the last few years, molecular techniques have dramatically improved the way IBDV is diagnosed (14, 15, 128). The reverse transcriptase polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (61, 68). Fresh bursa tissue can be submitted directly or bursas can be inactivated by phenol or FTA cards for molecular analysis (52-54, 99). After confirmation of the presence of IBDV by RT-PCR, further differentiation of the strains is possible with the use of restriction enzymes that can be used in a restriction fragment length polymorphism assay (RFLP) (55, 56, 100). DNA probes have also been used for determination of specific strains of IBDV. Most recently, nucleotide sequence analysis has been made readily available to most diagnostic laboratories. Many studies of the deduced amino acid sequences from the hypervariable region of VP2, has allowed for quicker diagnosis of IBDV strains than with previous diagnostic techniques (57).

Avian adeno-asociated virus as a vector.

- **Avian adeno-asociated virus.**

The use of the avian adeno-associated virus is a novel approach for the generation of a recombinant system for gene delivery in poultry that may offer distinct advantages over existing recombinant technologies currently used in the industry. The Parvoviruses are among the smallest of the DNA animal viruses. This family of viruses is composed of two subfamilies: the *Parvovirinae*, which infects vertebrates, and the *Densovirinae*, which infects insects. Each of these subfamilies have three genera: the *Parvovirus*, *Erythrovirus* and the *Dependovirus* are in the *Parvovirinae* subfamily, while the *Densovirus*, *Contravirus* and *Iteravirus* are in the *Densovirinae* subfamily (86, 87). The *Parvovirinae* has a wide distribution in warm-blooded animals, ranging from fowls to humans and the Dependoviruses are unique among animal viruses because, except under special conditions, they require a co-infection with an unrelated helper virus, namely an adenovirus or herpesvirus (77).

The parvovirus virions have a relatively simple structure, composed of a single-stranded DNA molecule encapsidated in a mixture of structural proteins, named VP1, VP2 and VP3, with an icosahedral symmetry of a T=1 arrangement. The major structural protein is the VP2 (90% of viral particle), which main structural motif is an eight-stranded antiparallel β-barrels, connected by large loops of amino acid chains. These loops form most of the capsid's surface and have important biological functions, such as host species and tissue tropism, receptor binding and antigenic properties (87). The viral particle has a molecular weight (MW) of 5.5 to 6.2 x 10^6 daltons. Approximately 50% of the mass is protein, and the remainder is DNA. Because of the

relatively high DNA-to-protein ratio, the buoyant density of the intact virion in cesium chloride (CsCl) is 1.39 to 1.42 g/cm3 (21). The heavy buoyant density in CsCl permits the ready separation of AAV from helper adenovirus (Ad) in co-infections. The sedimentation coefficient of the virion in neutral sucrose gradients is 110 to 122 (137). Possibly as a consequence of its structural simplicity, the virion is extremely resistant to inactivation. It is stable between pH 3 and 9 and at 56°C for 60 min. The virus can be inactivated by formalin, β-propriolactone, hydroxylamine, and oxidizing agents (90).

- **Parvovirus genetic map.**

The autonomous parvovirus genome contains two large open reading frames (ORFs). The first covers much of the left half of the genome and encodes two non structural (NS) proteins, NS1 and NS2, from alternately spliced mRNAs. Mutations within NS1 block viral replication and gene expression. Only NS1 is absolutely required for DNA replication (17). NS2- specific mutants are defective for capsid synthesis, gene expression, and DNA replication in murine cells but show variable phenotypes in human cells (87). The second large ORF occupies much of the right half of the genome and encodes the capsid proteins (90). Up to three capsid proteins have been detected in the virion. In the case of the minute virus of mice (MVM), the smallest capsid protein, VP3, is generated in the intact capsid by proteolytic cleavage of VP2. The amino acid sequences of VP1 and VP2 are identical except for additional amino acids at the N terminus of VP1 and they are synthesized from two alternatively spliced messages (87).

The *dependovirus* genetic map has been derived primarily from studies of the adenoassociated virus (AAV) type 2 but is highly conserved among all of the AAV serotypes

(42). There is a large ORF (cap) on the right side of the genome, which encodes the three coat proteins of the virus. There is also a large ORF in the left half of the genome, which has been called the rep region because any frameshift mutation or significant deletion within the region blocks DNA replication (42). These two ORFs are flanked by inverted terminal repeats (TR) that are palindromic in nature. At least four proteins have been detected (Rep78, Rep68, Rep52, and Rep40), which correspond to the four mRNAs that have been mapped to this region. In a productive infection, six 3' co-terminal polyadenylated and capped mRNAs can be detected by Northern blotting. These mRNAs are synthesized by the hosts RNA polymerase II from three different promoters designated p5, p19 and p40. The two largest transcripts are derived from p5 (4.2 and 3.9 kb in length), the next two largest transcripts are synthesized from p19 (3.6 and 3.3kb), while the two smallest RNAs (2.6 and 2.3 kb) are produced from p40 (87).

Mutations in either the rep or cap ORFs can be complemented *in trans*. In contrast, the inverted TRs of 145 bases are required *in cis* for DNA replication and transcription (20, 119). In addition to these functions, the TR is required for encapsidation (77), integration of the genome during the establishment of a latent infection (142), and rescue of the genome from the integrated state (114).

- **DNA Replication.**

The basic model for parvovirus DNA replication (often called the rolling hairpin model) applies to parvovirus replication. The palindromic inverted TR at the 3´ end of either strand can form a hairpin to serve as a primer to initiate synthesis of the complementary strand (73). This produces a linear duplex molecule in which the original 3´ end is covalently closed in the hairpin

configuration, also called a monomer turnaround form. The hairpin is nicked at a site near the end of the terminal palindrome (nucleotide 124 of the AAV sequence) and this produces a new 3′-hydroxyl primer that allows repair synthesis of the hairpin. This process is called terminal resolution (or strand transfer) and the site-specific and strand-specific nicking site is called the terminal resolution site (trs). The net result of terminal resolution is the first complete duplex molecule in which both ends are extended, the monomer extended form. Either end can then denature and reanneal to form a double hairpinned structure, or rabbit ears (a process sometimes called reinitiation) and the new hairpin formed at the 3′ end initiates leading-strand displacement synthesis to generate a single-stranded genome (which is packaged) and a duplex genome covalently closed at one end. The process of terminal resolution and reinitiation is then repeated. Each time the cycle is completed, a new single-stranded progeny strand is generated, and the strand transfer process that occurs during terminal resolution inverts the TR (87).

- **The Adeno Associated Virus**

One of the outstanding features of AAV replication in cell culture is the requirement for co-infection of the cell by an unrelated helper virus. Either an adeno (Ad) or a herpesvirus can supply complete helper functions for fully permissive AAV infection (84). In the absence of helper virus, AAV can establish a latent infection in cell culture that involves integration of the viral genome into a unique site on human chromosome 19. Infection of cell lines carrying a provirus with Ad or herpes will rescue the integrated genome and initiate a fully productive infection. In light of this, it has been assumed that AAV uses latency in chromosome 19 as a strategy to survive in the absence of a helper (24).

- **Helper Functions.**

Adenoviruses (Ad), herpes simplex virus (HSV) types I and II, cytomegalovirus (CMV), and pseudorabies virus all serve as complete helpers for AAV replication (87). The AAV host range is identical to the normal host range for the helper virus. Genetic analysis of helper functions has been most extensive for Ad. Many of the identified Ad early functions serve as helper functions for AAV replication. Four Ad proteins have been shown to be required for complete helper function: the early region IA (EIA) transactivator protein, the EIB 55-kd protein, the E4 34-kd protein, and the E2A DNA-binding protein. Additionally, synthesis of the Ad virus-associated (VA) RNAs is required (85). Similarly, an EIA function is required for AAV transcripts to be detected by Northern blotting (58).

- **The Avian Adeno Associated Virus.**

The Avian Adeno-Associated Virus (AAAV) was first isolated as a contaminant of isolates of the Olson strain of quail bronchitis virus (143). As with every member of the *dependovirus* genus, this viruses require a coinfection with a helper virus (an adeno or herpesvirus) to complete its own replication (87). Sadasiv and coworkers (113) postulated that the AAAV can exist as a latent infection in the germ line of chickens. In their study, chicken kidney cells derived from the eggs of white leghorn chickens that had serological evidence of prior exposure to both adenovirus and the AAAV, upon challenge with purified adenovirus, produced AAAV antigenic proteins. Another study that confirms these findings was performed by Dawson and Yates in 1982 (29). They demonstrated that AAAV could be consistently recovered from limiting dilutions of purified and unpurified avian Ad stocks propagated in embryonating chicken eggs derived from two independently raised flocks of White Leghorn (WL) chickens, but not when

these Ad stocks were propagated in duck cells. From these observations it was concluded that AAAV is a latent endogenous virus of at least some flocks of WL chickens.

Studies have demonstrated that there is genomic variability in AAAV isolates. Two strains of AAAV with very distinct DNA patterns upon restriction enzymes analysis have been found. Although the DNAs of both viruses characterized in this study had the same size, most restriction enzymes produced different restriction patterns. The immunofluorescence and immunodiffusion test showed that the two *dependoviruses* were serologically indistinguishable and therefore were regarded as two different strains of the same virus. These two strains were named as AAAV VR-865 (which was the first described isolate) and isolated AAAV DA-1 (43) .

Adeno-Associated Virus as Viral Vectors

Eight serotypes of AAV have been found in primates (AAV-1 to AAV-8). Type 2 is the best-characterized primate serotype, and it was the first AAV used for the development of vectors for gene transfer (77). Most of the vectors currently in use are derived from AAV-2, but vectors based on AAV-1 have been recently described (140). The AAV2- based vectors contain only the left and right ITRs, and 139 or 45 nucleotides of non-repeated AAV sequences adjacent to the right terminal repeat, respectively (77).

Samulski et al. (114) demostrated that all the cis-acting AAV functions required for replication and virion production are located within the ITR and the immediately adjacent 45 nucleotides. In the AAV-based vectors, the two viral open reading frames (ORFs), which code for the capsid proteins (VP1, 2, and 3) and the four nonstructural Rep proteins (Rep78/68 and

Rep52/40) are replaced with the transgene of interest and its promoter and then transfected into the producer cells, where the viral genes necessary for virus production and packaging of the vector genome are provided in trans by packaging plasmids and helper viruses/plasmids.

One disadvantage of AAV-based vectors is their size limitation: The optimal size for AAV vectors lies between 4.1 and 4.9 kb (32). Although larger genomes can be packaged, the packaging efficacy is sharply reduced and the maximal size of the vector genome (including the two ITRs) is 5.2 kb. Because of this size limitation, a dual vector, trans-splicing AAV vector system was developed (89), which allows the transduction of target cells and tissues with two AAV vectors that contain a split expression cassette.

AAV-Vector Packaging Strategies.

The basic approach of the AAV packaging system is the same as in other vectors: The vector plasmid contains the required cis-acting viral sequences necessary for packaging and integration and the transgene, while the packaging plasmids provide the viral components required for virus production in *Trans.* However, because AAV is a dependovirus, its propagation depends on the functions provided by a helper virus (adenovirus or herpesvirus). The initial studies with AAV vectors revealed that both the AAV *rep* and *cap* genes can be provided in trans in producer cells and this resulted in the first generation of packaging systems (77). In this system, the packaging plasmid, named pAAV/Ad (115), encodes the *rep* and *cap* genes, and the essential helper functions are provided by the adenovirus.

Transfection of the AAV packaging construct together with the vector plasmid into adenovirus-infected packaging/helper cells results in excision of the ITR flanked transgene from the vector plasmid, followed by its amplification and packaging into AAV capsids. This first generation of AAV packaging yielded up to 102 rAAV particles per cell, but there is a major disadvantage: the co-production of wild-type adenovirus along with the AAV vector particles. This may lead to a loss in the activity of the AAV particles. Given the fact that viral proteins derived from inactivated adenovirus preparations elicit a cytotoxic T lymphocyte (CTL) immune response at the site of injection, it is clear that this packaging system is suboptimal, at least for clinical use (60).

To overcome this problem, a packaging system was developed that is free of adenovirus (141). The adenoviral genes that provide essential helper functions during the productive life cycle of AAV are the E1A, E1B, E2A, E4, and VA RNA genes (85). By cloning of the E2A, E4, and VA RNA genes into a high-copy plasmid, Xiao et al. (139) generated a helper plasmid (pXX6) that contains all the essential helper genes but lacks the adenovirus structural and replication genes and the adenoviral terminal repeats.

Integration versus Episomal Persistence of rAAV.

Theoretically, site-specific integration would be a highly desirable feature of a gene therapy vector, as it would ensure long-term even life-long expression of the transgene and minimize the mutagenic and carcinogenic potential, major drawbacks of random integrating vectors based on retroviruses. Although the ITRs are the only genomic elements necessary for integration, efficient integration and site specificity require the presence of the viral Rep protein (134).

Because AAV vectors lack the Rep expression cassette, it comes as no surprise that AAV vectors integrate with low efficacy and low specificity into the host genome.

Integration of rAAV has been observed in dividing HeLa cells (142), as well as in nondividing human neuronal cell lines *in vitro* (138). Apart from integration into the host genome, the presence of episomal forms of rAAV has been demonstrated *in vivo*. Studies demonstrated the presence of AAV vector DNA in an episomal circular form in muscle and brain tissue transduced with AAV vectors (87). Because this form of episomal AAV DNA persists for up to 9 months, the episomal AAV DNA might be a major contributor to the long-term expression of transgenes delivered with AAV vectors (34).

REFERENCES

1. Abdel-Alim, G. A., and Y. M. Saif. Pathogenicity of embryo-adapted serotype 2 OH strain of infectious bursal disease virus in chickens and turkeys. Avian Dis. 46:1001-1006. 2002.

2. Al-Garib, S. O., A. L. J. Gielkens, E. Gruys, and G. Koch. Review of Newcastle disease virus with particular references to immunity and vaccination. Worl. Poul. Sci. J. 59:185-200. 2003.

3. Aldous, E. W., and D. J. Alexander. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Path. 30:117-128. 2001.

4. Aldous, E. W., M. S. Collins, A. McGoldrick, and D. J. Alexander. Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. Vet. Microbiol. 80:201-212. 2001.

5. Alexander, D. J., and P. Reeve. The proteins of Newcastle disease virus. 1. Structural proteins. Microb. 5:199-212. 1972.

6. Alexander, D. J., and G. Parsons. Protection of chickens against challenge with the variant virus responsible for Newcastle disease in 1984 by conventional vaccination. Vet. Rec. 118:176-177. 1986.

7. Alexander, D. J. The epidemiology and control of avian influenza and Newcastle disease. J. Comp. Pathol.112:105-126. 1995.

8. Alexander, D. J. Newcastle disease and other avian Paramyxovirus. In: A laboratory manual for the isolation and identification of avian pathogens., 4th ed. D. Swayne, Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M., ed. American Association of Avian Pathologists., Kennette Square, PA. pp 156-163. 1998.

9. Alexander, D. J., H. T. Morris, W. J. Pollitt, C. E. Sharpe, R. L. Eckford, R. M. Sainsbury, L. M. Mansley, R. E. Gough, and G. Parsons. Newcastle disease outbreaks in domestic fowl and turkeys in Great Britain during 1997. Vet. Rec. 143:209-212. 1998.

10. Alexander, D. J. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Tech. 19:443-462. 2000.

11. Alexander, D. J. Gordon Memorial Lecture. Newcastle disease. Br. Poult. Sci. 42:5-22. 2001.

12. Alexander, D. J. Newcastle disease and other Paramyxoviridae infections. In: Diseases of Poultry., 11th ed. Y. M. Saif, Barnes, H., Glisson, I., Fadly, A., McDouglad, J. and Swayne, D., ed. Iowa State Press., Ames, IA. pp 63-87. 2003.

13. Allan, W. H., and R. E. Gough. A standard haemagglutination inhibition test for Newcastle disease. (1). A comparison of macro and micro methods. Vet. Rec. 95:120-123. 1974.

14. Banda, A., P. Villegas, J. El-Attrache, and C. Estevez. Molecular characterization of seven field isolates of infectious bursal disease virus obtained from commercial broiler chickens. Avian Dis. 45:620-630. 2001.

15. Banda, A., P. Villegas, and J. El-Attrache. Molecular characterization of infectious bursal disease virus from commercial poultry in the United States and Latin America. Avian. Dis. 47:87-95. 2003.

16. Banda, A., and P. Villegas. Genetic characterization of very virulent infectious bursal disease viruses from Latin America. Avian. Dis. 48:540-549. 2004.

17. Bartlett, J. S., R. Wilcher, and R. J. Samulski. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J. Virol. 74:2777-2785. 2000.

18. Bayliss, C. D., U. Spies, K. Shaw, R. W. Peters, A. Papageorgiou, H. Muller, and M. E. Boursnell. A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. J. Gen. Virol. 71:1303-1312. 1990.

19. Beard, C. W., P. Villegas, and J. R. Glisson. Comparative efficacy of the B-1 and VG/GA vaccine strains against velogenic viscerotropic Newcastle disease virus in chickens. Avian Dis. 37:222-225. 1993.

20. Beaton, A., P. Palumbo, and K. I. Berns. Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated *in trans* by the rep protein. J. Virol. 63:4450-4454. 1989.

21. Berns, K. I., and R. A. Bohenzky. Adeno-associated viruses: an update. Adv. Virus. Res. 32:243-306. 1987.

22. Brown, C., D. J. King, and B. S. Seal. Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Vet. Pathol. 36:125-132. 1999.

23. Chettle, N. J., C. Stuart, P.J. Wyeth. Outbreaks of virulent infectious bursal disease in East Anglia. Vet. Rec.271-272. 1989.

24. Cheung, A. K., M. D. Hoggan, W. W. Hauswirth, and K. I. Berns. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. 33:739-748. 1980.

25. Collins, M. S., I. Strong, and D. J. Alexander. Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses". Arch. Virol. 134:403-411. 1994.

26. Cosgrove, A. S. An apparently new disease of chickens: Avian nephrosis. Avian Dis. 6:385-389. 1962.

27. Coulibaly, F., C. Chevalier, I. Gutsche, J. Pous, J. Navaza, S. Bressanelli, B. Delmas, and F. A. Rey. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell 120:761-772. 2005.

28. Craft, D. W., J. Brown, and P. D. Lukert. Effects of standard and variant strains of infectious bursal disease virus on infections of chickens. Am. J. Vet. Res. 51:1192-1197. 1990.

29. Dawson, G. J., V. J. Yates, P. W. Chang, and J. J. Oprandy. Is avian adeno-associated virus an endogenous virus of chicken cells? Nature 298:580-582. 1982.

30. de Leeuw, O. S., L. Hartog, G. Koch, and B. P. Peeters. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. J. Gen. Virol. 84:475-484. 2003.

31. de Leeuw, O. S., G. Koch, L. Hartog, N. Ravenshorst, and B. P. Peeters. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J. Gen. Virol. 86:1759-1769. 2005.

32. Dong, J. Y., P. D. Fan, and R. A. Frizzell. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. Hum. Gene. Ther. 7:2101-2112. 1996.

33. Dormitorio, T. V., J. J. Giambrone, and L. W. Duck. Sequence comparisons of the variable VP2 region of eight infectious bursal disease virus isolates. Avian Dis. 41:36-44. 1997.

34. Duan, D., P. Sharma, J. Yang, Y. Yue, L. Dudus, Y. Zhang, K. J. Fisher, and J. F. Engelhardt. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. J. Virol. 72:8568-8577. 1998.

35. Erf, G. F. Cell-mediated immunity in poultry. Poult. Sci. 83:580-590. 2004.

36. Ewert, D. L., B. O. Barger, and C. S. Eidson. Local antibody response in chickens: analysis of antibody synthesis to Newcastle disease virus by solid-phase radioimmunoassay and immunofluorescence with class-specific antibody for chicken immunoglobulins. Infect. Immun. 24:269-275. 1979.

37. Faragher, J. T. Infectious bursal disease of chicken. Vet. Bull.361-369. 1972.

38. Glickman, R. L., R. J. Syddall, R. M. Iorio, J. P. Sheehan, and M. A. Bratt. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J. Virol. 62:354-356. 1988.

39. Gohm, D. S., B. Thur, and M. A. Hofmann. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. Avian Path. 29:143-152. 2000.

40. Hairul Aini, H., A. R. Omar, M. Hair-Bejo, and I. Aini. Comparison of Sybr Green I, ELISA and conventional agarose gel-based PCR in the detection of infectious bursal disease virus. Microbiol. Res. 2006.

41. Hanson, R. P., and C. A. Brandly. Identification of vaccine strains of Newcastle disease virus. Science 122:156-157. 1955.

42. Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. 51:329-339. 1984.

43. Hess, M., G. Paul, S. Kling, and G. Monreal. Molecular characterization of two strains of the avian adeno-associated virus (AAAV). Arch. Virol. 140:591-598. 1995.

44. Hirai, K., and S. Shimakura. Structure of infectious bursal disease virus. J. Virol. 14:957-964. 1974.

45. Hirai, K., S. Shimakura, and E. Kawamoto. Electron-microscope characterization of infectious bursal disease virus. Avian Dis. 18:467-471. 1974.

46. Hirai, K., K. Kunihiro, and S. Shimakura. Characterization of Immunosuppression in chickens by infectious bursal disease virus. Avian Dis. 23:950-965. 1979.

47. Hitchner, S. B. Infectivity of infectious bursal disease virus for embryonating eggs. Poult. Sci. 49:511-516. 1970.

48. Hitchner, S. B. Persistence of parental infectious bursal disease antibody and its effect on susceptibility of young chickens. Avian Dis. 15:894-900. 1971.

49. Howie, R., and J. Thorsen. An enzyme-linked immunosorbent assay (ELISA) for infectious bursal disease virus. Can. J. Comp. Med. 45:51-55. 1981.

50. Huang, Z., S. Krishnamurthy, A. Panda, and S. K. Samal. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J. Virol. 77:8676-8685. 2003.

51. Huang, Z., A. Panda, S. Elankumaran, D. Govindarajan, D. D. Rockemann, and S. K. Samal. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. J. Virol. 78:4176-4184. 2004.

52. Jackwood, D. J., and R. J. Jackwood. Infectious bursal disease viruses: molecular differentiation of antigenic subtypes among serotype 1 viruses. Avian Dis. 38:531-537. 1994.

53. Jackwood, D. J., G. Hanes, and S. H. Miller. Infectious bursal disease viral RNA amplification using RT/PCR from bursa tissue following phenol: chloroform inactivation of the virus. Avian Dis. 40:457-460. 1996.

54. Jackwood, D. J., and R. J. Jackwood. Molecular identification of infectious bursal disease virus strains. Avian Dis. 41:97-104. 1997.

55. Jackwood, D. J., and S. E. Sommer. Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses. Avian Dis. 41:627-637. 1997.

56. Jackwood, D. J., S. E. Sommer, and H. V. Knoblich. Amino acid comparison of infectious bursal disease viruses placed in the same or different molecular groups by RT/PCR-RFLP. Avian Dis. 45:330-339. 2001.

57. Jackwood, D. J., B. D. Spalding, and S. E. Sommer. Real-time reverse transcriptase-polymerase chain reaction detection and analysis of nucleotide sequences coding for a neutralizing epitope on infectious bursal disease viruses. Avian Dis. 47:738-744. 2003.

58. Janik, J. E., M. M. Huston, K. Cho, and J. A. Rose. Efficient synthesis of adeno-associated virus structural proteins requires both adenovirus DNA binding protein and VA I RNA. Virol. 168:320-329. 1989.

59. Jayawardane, G. W., and P. B. Spradbrow. Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. Vet. Microbiol. 46:69-77. 1995.

60. Kafri, T., D. Morgan, T. Krahl, N. Sarvetnick, L. Sherman, and I. Verma. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: Implications for gene therapy. Proceed. Nat. Acad. Sci U.S.A 95:11377-11382. 1998.

61. Kataria, R. S., A. K. Tiwari, T. Nanthakumar, and P. P. Goswami. One-step RT-PCR for the detection of infectious bursal disease virus in clinical samples. Vet. Res. Commun. 25:429-436. 2001.

62. King, D. J. Avian paramyxovirus type 1 from pigeons: isolate characterization and pathogenicity after chicken or embryo passage of selected isolates. Avian Dis. 40:707-714. 1996.

63. Kommers, G. D., D. J. King, B. S. Seal, and C. C. Brown. Virulence of pigeon-origin Newcastle disease virus isolates for domestic chickens. Avian Dis. 45:906-921. 2001.

64. Kommers, G. D., D. J. King, B. S. Seal, K. P. Carmichael, and C. C. Brown. Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. Vet. Pathol. 39:353-362. 2002.

65. Kommers, G. D., D. J. King, B. S. Seal, and C. C. Brown. Virulence of six heterogeneous-origin Newcastle disease virus isolates before and after sequential passages in domestic chickens. Avian Pathol. 32:81-93. 2003.

66. Lamb, R. a. P., G. Paramyxoviridae: The Viruses and their Replication. In: Fields Virology. D. a. H. P. E. Knipe, ed. Lipincott Williams & Wilkins., Philadelphia, PA. pp 1499-1496. 2007.

67. Lasher, H. N., and V. S. Davis. History of infectious bursal disease in the U.S.A.--the first two decades. Avian Dis. 41:11-19. 1997.

68. Lee, L. H., S. L. Yu, and H. K. Shieh. Detection of infectious bursal disease virus infection using the polymerase chain reaction. J. Virol. Methods 40:243-253. 1992.

69. Leslie, J. Newcastle disease: outbreak losses and control policy costs. Vet. Rec. 146:603-606. 2000.

70. Liu, M., and V. N. Vakharia. VP1 protein of infectious bursal disease virus modulates the virulence in vivo. Virol. 330:62-73. 2004.

71. Locke, D. P., H. S. Sellers, J. M. Crawford, S. Schultz-Cherry, D. J. King, R. J. Meinersmann, and B. S. Seal. Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. Virus Res. 69:55-68. 2000.

72. Lukert, P. D. a. Y. M. S. Infectious Bursal Disease. In: Diseases of Poultry, 10th ed. H. J. B. B.W. Calnek, C.W. Beard, L.R. McDougald, Y.M. Saif ed. Iowa State University Press, Ames, IA. pp 721-738. 1997.

73. Lusby, E., K. H. Fife, and K. I. Berns. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. J. Virol. 34:402-409. 1980.

74. Marin, M. C., P. Villegas, J. D. Bennett, and B. S. Seal. Virus characterization and sequence of the fusion protein gene cleavage site of recent Newcastle disease virus field isolates from the southeastern United States and Puerto Rico. Avian Dis. 40:382-390. 1996.

75. Marquardt, W. W., R. B. Johnson, W. F. Odenwald, and B. A. Schlotthober. An indirect enzyme-linked immunosorbent assay (ELISA) for measuring antibodies in chickens infected with infectious bursal disease virus. Avian Dis. 24:375-385. 1980.

76. Mayo, M. A. Virus taxonomy - Houston 2002. Arch. Virol. 147:1071-1076. 2002.

77. McLaughlin, S. K., P. Collis, P. L. Hermonat, and N. Muzyczka. Adeno-associated virus general transduction vectors: analysis of proviral structures. J. Virol. 62:1963-1973. 1988.

78. Mebatsion, T., S. Verstegen, L. T. De Vaan, A. Romer-Oberdorfer, and C. C. Schrier. A recombinant newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. J. Virol. 75:420-428. 2001.

79. Muller, H., C. Scholtissek, and H. Becht. The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. J. Virol. 31:584-589. 1979.

80. Muller, H., and R. Nitschke. Molecular weight determination of the two segments of double-stranded RNA of infectious bursal disease virus, a member of the birnavirus group. Med. Microbiol. Immunol.176:113-121. 1987.

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

82. Mundt, E., J. Beyer, and H. Muller. Identification of a novel viral protein in infectious bursal disease virus-infected cells. J. Gen. Virol. 76:437-443. 1995.

83. Mundt, E., B. Kollner, and D. Kretzschmar. VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. J. Virol. 71:5647-5651. 1997.

84. Muzyczka, N., R. J. Samulski, P. Hermonat, A. Srivastava, and K. I. Berns. The genetics of adeno-associated virus. Adv. Exp. Med. Biol. 179:151-161. 1984.

85. Muzyczka, N. Use of adeno-associated virus as a general transduction vector for mammalian cells. Curr. Top. Microbiol. Immunol. 158:97-129. 1992.

86. Muzyczka, N. Adeno-associated virus (AAV) vectors: will they work? J. Clin. Invest. 94:1351. 1994.

87. Muzyczka, N. a. K. B. Parvoviridae: the viruses and their replication. In: Fields Virology. D. a. H. P. E. Knipe, ed. Lippincott-Raven., Philadelphia. pp 2327-2379. 2001.

88. Nagai, Y. A molecular basis for the variations in virulence of Newcastle disease virus. Tanpakushitsu. Kakusan. Koso. 22:385-395. 1977.

89. Nakai, H., T. A. Storm, and M. A. Kay. Increasing the size of rAAV-mediated expression cassettes in vivo by intermolecular joining of two complementary vectors. Nat. Biotechnol. 18:527-532. 2000.

90. Nam, H. J., M. D. Lane, E. Padron, B. Gurda, R. McKenna, E. Kohlbrenner, G. Aslanidi, B. Byrne, N. Muzyczka, S. Zolotukhin, and M. Agbandje-McKenna. Structure of Adeno-Associated virus serotype 8, a gene therapy vector. J. Virol. 2007.

91. Nunes, J. E. S., A. C. Vasconcelos, M. A. Jorge, E. B. Guimaraes, T. A. Paixao, N. R. S. Martins, and J. S. Resende. Comparative morphometric analysis of vaccinal virulence of some lentogenic strains of Newcastle disease virus in tracheas of SPF chickens. Arq. Bras. .Med. Vet. Zoot.54:335-339. 2002.

92. Nunoya, T., Y. Otaki, M. Tajima, M. Hiraga and T. Saito. Occurence of acute infectious bursal disease with high mortality in Japan and pathogenicity of field isolates in SPF chickens. Avian Dis.596-609. 1992.

93. Panda, A., Z. Huang, S. Elankumaran, D. D. Rockemann, and S. K. Samal. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. Microb. Pathog. 36:1-10. 2004.

94. Pardo, M. Characterizations of the lentogenic Newcastle disease virus strain VG/GA. Master Thesis Athens, GA. University of Georgia. USA. pp.132. 1991.

95. Pardo, M., P. Villegas. VG/GA strain of Newcastle disease virus strain. 1992.

96. Peeters, B. P., O. S. de Leeuw, G. Koch, and A. L. Gielkens. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J. Virol. 73:5001-5009. 1999.

97. Perozo, F., P. Villegas, C. Estevez, I. Alvarado, and L. B. Purvis. Use of FTA filter paper for the molecular detection of Newcastle disease virus. Avian. Pathol. 35:93-98. 2006.

98. Perozo, F., Nava, J. & Rivera, S. Evaluation of two vaccination programs against Newcastle disease in Ross line broiler chickens reared under field conditions in Zulia state, Venezuela. 2. Immune response and protection against an experimental challenge. Revista cientifica FCV/LUZ 14:387-394. 2004.

99. Purvis, L. B., P. Villegas, and F. Perozo. Evaluation of FTA paper and phenol for storage, extraction and molecular characterization of infectious bursal disease virus. J. Virol. Methods 138:66-69. 2006.

100. Qian, B., and F. S. Kibenge. Restriction fragment profiles of genome segment A of infectious bursal disease virus correlate with serotype and geographical origin of avibirnaviruses. Can. J. Microbiol. 42:93-97. 1996.

101. Remorini, P., M. G. Calderon, S. Aguirre, O. Periolo, J. La Torre, and N. Mattion. Characterization of infectious bursal disease viruses from Argentina. Avian Dis. 50:245-251. 2006.

102. Reynolds, D. L., and A. D. Maraqa. Protective immunity against Newcastle disease: the role of antibodies specific to Newcastle disease virus polypeptides. Avian Dis. 44:138-144. 2000.

103. Reynolds, D. L., and A. D. Maraqa. Protective immunity against Newcastle disease: the role of cell-mediated immunity. Avian Dis. 44:145-154. 2000.

104. Romer-Oberdorfer, A., E. Mundt, T. Mebatsion, U. J. Buchholz, and T. C. Mettenleiter. Generation of recombinant lentogenic Newcastle disease virus from cDNA. J. Gen. Virol. 80: 2987-2995. 1999.

105. Romer-Oberdorfer, A., O. Werner, J. Veits, T. Mebatsion, and T. C. Mettenleiter. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J. Gen. Virol. 84:3121-3129. 2003.

106. Rosenberger, J. K., S. Klopp, R. J. Eckroade, and W. C. Krauss. The roles of the infectious bursal agent and several avian adenoviruses in the hemorrhagic-aplastic-anemia syndrome and gangrenous dermatitis. Avian Dis. 19:717-729. 1975.

107. Rosenberger, J. K., and J. Gelb. Immunosuppressive effects of the infectious bursal agent and relationships to other poultry diseases. Proc. Annu. Meet. U S. Anim. Health. Assoc.:283-289. 1976.

108. Rosenberger, J. K., S. S. Cloud, J. Gelb Jr., E. Odor, and J.E. Dohms Sentinel bird survey of Delmarva broiler flocks. In: In Proc. 20th Natl. Meet. Poult. Health .Condemn. Ocean City. pp 94-101. 1985.

109. Rosenberger, J. K., Y.M. Saif, and D.J. Jackwood Infectious Bursal Disease. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. J. R. G. D.E. Swayne, M.W. Jackwood, J.E. Pearson, W.M. Reed, ed. American Association of Avian Pathologists Kennett Square, PA. pp 215-218. 1998.

110. Rosenberger, J. K. a. S. S. C. Isolation and Characterization of variant infectious bursal disease virus. J. Am. Vet. Med. Assoc. 189:357. 1986.

111. Russell, P. H. Newcastle disease virus: virus replication in the harderian gland stimulates lacrimal IgA; the yolk sac provides early lacrimal IgG. Vet. Immunol. Immunopathol. 37:151-163. 1993.

112. Russell, P. H., and G. Koch. Local antibody forming cell responses to the Hitchner B1 and Ulster strains of Newcastle disease virus. Vet. Immunol. Immunopathol. 37:165-180. 1993.

113. Sadasiv, E. C., T. H. Piela, and P. W. Chang. Detection of latent avian adenovirus-associated virus proteins in chicken cells. Avian Dis. 33:125-133. 1989.

114. Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell 33:135-143. 1983.

115. Samulski, R. J., L. S. Chang, and T. Shenk. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. 63:3822-3828. 1989.

116. Scott, T. R. Our current understanding of humoral immunity of poultry. Poult. Sci. 83:574-579. 2004.

117. Seal, B. S., D. J. King, and J. D. Bennett. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. J. Clin. Microbiol. 33:2624-2630. 1995.

118. Seal, B. S., D. J. King, and H. S. Sellers. The avian response to Newcastle disease virus. Dev. Comp. Immunol. 24:257-268. 2000.

119. Senapathy, P., J. D. Tratschin, and B. J. Carter. Replication of adeno-associated virus DNA. Complementation of naturally occurring rep- mutants by a wild-type genome or an ori-mutant and correction of terminal palindrome deletions. J. Mol. Biol. 179:1-20. 1984.

120. Sharma, J. M., J. E. Dohms, and A. L. Metz. Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. Avian Dis. 33:112-124. 1989.

121. Snyder, D. B., D. P. Lana, B. R. Cho, and W. W. Marquardt. Group and strain-specific neutralization sites of infectious bursal disease virus defined with monoclonal antibodies. Avian Dis. 32:527-534. 1988.

122. Snyder, D. B., D. P. Lana, P. K. Savage, F. S. Yancey, S. A. Mengel, and W. W. Marquardt. Differentiation of infectious bursal disease viruses directly from infected tissues with

neutralizing monoclonal antibodies: evidence of a major antigenic shift in recent field isolates. Avian Dis. 32:535-539. 1988.

123. Snyder, D. B. Changes in the field strains of infectious bursal disease virus. Avian Path. 419-423. 1990.

124. Spies, U., H. Muller, and H. Becht. Nucleotide sequence of infectious bursal disease virus genome segment A delineates two major open reading frames. Nucleic. Acids. Res. 17:7982. 1989.

125. Steward, M., I. B. Vipond, N. S. Millar, and P. T. Emmerson. RNA editing in Newcastle disease virus. J. Gen. Virol. 74:2539-2547. 1993.

126. Takimoto, T., G. L. Taylor, S. J. Crennell, R. A. Scroggs, and A. Portner. Crystallization of Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. Virol.270:208-214. 2000.

127. Thayer, S. G., B. N. Nersessian, B. Rivetz, and O. J. Fletcher. Comparison of serological tests for antibodies against Newcastle disease virus and infectious bronchitis virus using ImmunoComb solid-phase immunoassay, a commercial enzyme-linked immunosorbent assay, and the hemagglutination-inhibition assay. Avian Dis. 31:459-463. 1987.

128. Vakharia, V. N., J. He, B. Ahamed, and D. B. Snyder. Molecular basis of antigenic variation in infectious bursal disease virus. Virus Res. 31:265-273. 1994.

129. Van den Berg, T. P., P.M. Gonze, and G. Meulemans. Acute infectious bursal disese in poultry: isolation and charcterization of a highly virulent strain. Avian Path.133-143. 1991.

130. Van der Marel, P., D. Snyder, and D. Lutticken. Antigenic characterization of IBDV field isolates by their reactivity with a panel of monoclonal antibodies. Avian Path. 97:81-83. 1990.

131. Villegas, P. Viral diseases of the respiratory system. Poult. Sci. 77:1143-1145. 1998.

132. von Einem, U. I., A. E. Gorbalenya, H. Schirrmeier, S. E. Behrens, T. Letzel, and E. Mundt. VP1 of infectious bursal disease virus is an RNA-dependent RNA polymerase. J. Gen. Virol. 85:2221-2229. 2004.

133. Wakamatsu, N., D. J. King, B. S. Seal, B. P. Peeters, and C. C. Brown. The effect on pathogenesis of Newcastle disease virus LaSota strain from a mutation of the fusion cleavage site to a virulent sequence. Avian Dis. 50:483-488. 2006.

134. Weitzman, M. D., S. R. Kyostio, R. M. Kotin, and R. A. Owens. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proc. Natl. Acad. Sci. U.S.A 91:5808-5812. 1994.

135. Winterfield, R. W., A. M. Fadly, and A. Bickford. Infectivity and distribution of infectious bursal disease virus in the chicken. Persistence of the virus and lesions. Avian Dis. 16:622-632. 1972.

136. Winterfield, R. W., S.B. Hitchner, G.S. Appleton, and A.S. Cosgrove. Avian nephrosis, nephritis and Gumboro disease. L & M News Views 3:103. 1962.

137. Wistuba, A., S. Weger, A. Kern, and J. A. Kleinschmidt. Intermediates of adeno-associated virus type 2 assembly: identification of soluble complexes containing Rep and Cap proteins. J. Virol. 69:5311-5319. 1995.

138. Wu, P., M. I. Phillips, J. Bui, and E. F. Terwilliger. Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. J. Virol. 72:5919-5926. 1998.

139. Xiao, W., S. C. Berta, M. M. Lu, A. D. Moscioni, J. Tazelaar, and J. M. Wilson. Adeno-associated virus as a vector for liver-directed gene therapy. J. Virol. 72:10222-10226. 1998.

140. Xiao, W., N. Chirmule, S. C. Berta, B. McCullough, G. Gao, and J. M. Wilson. Gene therapy vectors based on adeno-associated virus type 1. J Virol. 73:3994-4003. 1999.

141. Xiao, X., J. Li, and R. J. Samulski. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J. Virol. 72:2224-2232. 1998.

142. Yang, C. C., X. Xiao, X. Zhu, D. C. Ansardi, N. D. Epstein, M. R. Frey, A. G. Matera, and R. J. Samulski. Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro. J. Virol. 71:9231-9247. 1997.

143. Yates, V. J., A. M. el-Mishad, K. J. McCormick, and J. J. Trentin. Isolation and characterization of an Avian adenovirus-associated virus. Infect. Immun. 7:973-980. 1973.

144. Yusoff, K., and W. S. Tan. Newcastle disease virus: macromolecules and opportunities. Avian Pathol. 30:439-455. 2001.

145. Zeng, J., P. Fournier, and V. Schirrmacher. High cell surface expression of Newcastle disease virus proteins via replicon vectors demonstrates syncytia forming activity of F and fusion promotion activity of HN molecules. Int. J. Oncol. 25:293-302. 2004.

146. Zigterman, G. J., W. van de Ven, C. van Geffen, A. H. Loeffen, J. H. Panhuijzen, E. O. Rijke, and A. N. Vermeulen. Detection of mucosal immune responses in chickens after immunization or infection. Vet. Immunol. Immunopathol. 36:281-291. 1993.

CHAPTER III

AVIAN ADENO-ASSOCIATED VIRUS BASED EXPRESSION OF NEWCASTLE DISEASE VIRUS HEMAGGLUTININ-NEURAMINIDASE PROTEIN FOR POULTRY VACCINATION [1].

---

[1] F. Perozo, P. Villegas, C. Estevez, I. R. Alvarado, L. B. Purvis, E. Saume. **Accepted by Avian Diseases.**
Reprinted here with permission of the publisher**.** 02/02/2008.

SUMMARY.

The avian adeno-associated virus (AAAV) is a replication defective non-pathogenic virus member of the family *Parvoviridae* that has been proved to be useful as a viral vector for gene delivery. The use of AAAV for transgenic expression of Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) protein and its ability to induce immunity in chickens was assessed. Proposed advantages of this system include: no interference with maternal antibodies, diminished immune response against the vector and the ability to accommodate large fragments of genetic information. In this work, the generation of recombinant AAAV virions expressing the HN protein (rAAAV-HN) was demonstrated by electron microscopy, immunocytochemistry and western blot analysis. Serological evidence of HN protein expression after *in ovo* or intramuscular inoculation of the recombinant virus in specific pathogen free (SPF) chickens was obtained. Serum from rAAAV-HN vaccinated birds showed a systemic immune response evidenced by NDV specific enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) testing. Positive virus neutralization in embryonated chicken eggs and indirect immunofluorescense (IF) detection of NDV infected cells by serum from rAAAV-HN vaccinated birds is also reported. A vaccine-challenge experiment in commercial broiler chickens using a Venezuelan virulent viscerotropic strain of NDV was performed. All unvaccinated controls died within five days post-challenge. Protection up to 80% was observed in birds vaccinated *in ovo* and revaccinated at seven days of age with the rAAAV-HN. The results demonstrate the feasibility of developing and using an avian adeno-associated virus based gene delivery system for poultry vaccination.

Key words: Avian adeno-associated virus, vaccination, immune response, Newcastle disease virus.

Newcastle disease virus (NDV) is a single stranded negative sense RNA virus member of the *Paramyxoviridae* family in the genus *Avulavirus* (13). Due to variation in viral virulence and in susceptibility of hosts, the symptoms of NDV in infection in domestic species (chicken, turkey, goose, duck and pigeon) range from unapparent to severe, the later including respiratory, enteric and nervous system disease (2). The NDV infection of host cells is accomplished by the interaction of two surface glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins (3, 12). The HN protein possesses both receptor recognition of sialic acid at the termini of host glycoconjugates and neuraminidase activity to hydrolyze sialic acid from progeny virion particles to prevent viral self-aggregation (2, 22). In addition to these activities, the HN protein has been shown to promote fusion through its interaction with the F protein, thereby allowing the entry of viral RNA (12, 30). The HN is also readily recognized by the host immune response as a major antigenic protein (19).

Vaccination of commercially reared birds is the best way to reduce losses resulting from NDV infection (1). Live or inactivated vaccines currently used in most countries for NDV control are derived from lentogenic field strains (31). Live attenuated vaccines are infectious and the inactivated vaccines may result in disease emergence due to lack of antigenic stimulation at the natural site of virus entry. In addition, inactivated vaccines are unable to replicate, they do not elicit protein production in the cell and hence viral antigens cannot be presented by MHC class I molecules, thus cytotoxic CD8 T cells are not generated (1, 23, 29). To avoid some of these limitations, the poultry industry has resorted to the use of recombinant viruses for infectious diseases control; the advantages of the recombinant technology for poultry vaccination include no reversion in pathogenicity and absence of vaccine reactions (10, 14, 17, 18).

The use of replication defective parvoviruses for the purpose of gene delivery is a leading trend in human medicine, as demonstrated up to 2005 when at least twenty clinical trials using fifteen different adeno-associated viruses (AAV) vectors had been completed or were ongoing (25). These replication defective parvoviruses are non-pathogenic, capable of accommodating relatively long pieces of DNA and of infecting a wide variety of cell types (11, 16, 24). A member of this family, the avian adeno-associated virus (AAAV) has been fully characterized (6, 8) and successfully used for reporter gene delivery in chicken embryo cells (9). Proposed advantages of this viral vector include: no interference of maternal antibodies, diminished immune response against the vector and the ability to accommodate large fragments of genetic information (9, 16, 24). The possibility of using recombinant AAAV for transgenic expression of immunogenic proteins for poultry vaccination is to be assessed. The aim of this work was the generation of recombinant AAAV virions expressing the immunogenic HN protein of LaSota strain of NDV and to assess their ability to generate protective immunity in chickens.

MATERIALS AND METHODS.

Amplification of the HN gene by reverse transcriptase-polymerase chain reaction (RT-PCR). LaSota strain of Newcastle disease was obtained from the viral stock of the Poultry Diagnostic and Research Center of the University of Georgia and used to amplify the 1341 bp of the HN gene using the following primers: Forward HN CDS F (5'-AGCATCGATATGGACCGCGCCGTTA-3') and reverse HN CDS R (5'-AGCAGATCTTTACTAGCCAGACCTGGCTTCTCTA-3'). To obtain directional insertion with respect of the cytomegalovirus (CMV) major late promoter located in the expression plasmid, the *Cla*I and *Bgl*II restriction sites were included in the 5'end of the forward and reverse primers, respectively. Reverse transcription was performed with the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) while polymerase chain reactions (PCR) were carried out with the FailSafe PCR system (Epicentre, Madison, WI) following the manufacturers' instructions. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide with a concentration of 0.5 μg/ml. The products of the proper size were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) as indicated by the manufacturer.

Cloning and sequencing of the HN gen.

The amplified HN gene was cloned in pCR 2.1 plasmids (Invitrogen, Carlsbad, CA) and transformed into TOP 10 *E. coli* cells. Positive colonies were selected and recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The presence of the HN gene was corroborated by digestion of these plasmids with the *Cla*I and *Bgl*II restriction enzymes and electrophoresis analysis. Direct nucleotide sequencing of the HN gene with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was

performed. When compared with reference HN (AF077761) available at GenBank, two non-synonymous mutations in the HN gene introduced during the reverse transcription reaction were detected (G – 566 – A, and G – 1357 – A).

Site-directed mutagenesis.

The point mutations were reversed by using the QuikChange and QuikChange Multi Site-Directed Mutagenesis kits (Stratagene, La Jolla, CA) following manufacturer's instructions. After site-directed mutagenesis, the HN gene was sequenced showing 100% similarity with the reference strain.

Generation of plasmid constructs to produce recombinant AAAV particles coding for the HN gene. DNA fragments of 1341 bp (HN gene) recovered from the pCR 2.1 plasmid were cloned downstream of the CMV promoter in a previously developed construct named p3.6 ITR-MCS containing the inverted terminal repeats (ITR) of the DA-1 strain of AAAV (8). Briefly, the p3.6 ITR-MCS plasmid was linearized with the *Cla*I and *Bgl*II restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Inc., Beverly, MA) at 37 C x 30 min. Equimolar concentrations of the HN gene were ligated using T4 DNA ligase and transfected in TOP 10 *E. coli* cells generating a plasmid construct designated pAAAV-NDV-HN (Fig. 1). Positive transformed colonies were selected, grown overnight at 37 °C in selective Luria Bertani medium with ampicillin and purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The presence of the HN gene in the p3.6 ITR-MCS was verified by electrophoresis after digestion with the *Cla*I and *Bgl*II restriction enzymes.

Expression of the HN protein. Monolayers of human embryo kidney cell line 293 (HEK 293) obtained from ATCC (CRL-1573) were grown using Dulbecco's modified minimal essential medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics in 25

cm^2 cell culture flasks and subcultured in 35 mm^2 plates until 90% confluence. Ten micrograms of the p3.6 ITR-MCS plasmid containing the HN gene (pAAAV-NDV-HN) were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The expression of the HN proteins was detected 72 hr post-transfection by immunocytochemistry using the Diaminobenzidine-Urea-Peroxidase complex (Sigma Chemicals Co., St Louis, MO) following the manufacturer's instructions. Commercial chicken NDV specific antiserum (SPAFAS Inc., Norwich, CT) was used on transfected cells and non-transfected controls. The presence of positive brown staining in the transfected HEK 293 cells was considered positive for HN expression.

Generation of recombinant AAAV virions expressing the HN proteins. At the vector core facility in the gene therapy center of the University of North Carolina (Chapel Hill, NC), the pAAAV-NDV-HN, a plasmid named pRC-CMV expressing the Rep and Cap coding regions of the DA-1 strain of AAAV (8) and a commercial pHelper plasmid coding for the E2, E4 and VR-RNA genes derived from the human adenovirus type 5 (Stratagene, La Jolla, CA) were simultaneously transfected in HEK293 cells to produce the rAAAV-HN virions following their standard procedures. The titer for the rAAAV-HN obtained was determined by dot blot and reported as virus molecules/ml: 2.5×10^{11}. The generation of the rAAAV-HN virions was confirmed by immunocytochemistry as explained above and by electron microscopy as explained elsewhere (5).

Western blot. To evaluate whether the protein expression induced by the rAAAV-HN virions generates a HN protein of the appropriate size, western blot analysis of infected chicken embryo fibroblasts (CEF) cells was performed. Briefly, CEF monolayers were infected with the rAAAV-HN virions, LaSota strain of NDV or with the wild type AAAV strain DA-1 and incubated for 48

h at 37°C. Lysates of infected and uninfected cells were separated by SDS/PAGE and transferred to nitrocellulose filters (TransBlot SD cell; Bio-Rad). Blots were incubated with monoclonal NDV HN specific mouse antiserum (NDV-60) provided by Dr. Egbert Mundt from the University of Georgia. Binding of peroxidase-conjugated mouse-specific secondary antibody (KPL Kierkegaard & Perry Lab. USA) was detected by chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotecnology, Rockford. IL).

Serological evidence of the HN protein expression. Specific pathogen free (SPF) embryonated chicken eggs (Sunrise Farms. Catskil. NY) were divided in six groups of 20 eggs and used to test the serological response to two different doses: 10^8 or 10^9 virus molecules/0.1 ml and two administration routes: *in ovo* at the 18^{th} day of incubation or intramuscular inoculation of one day-old chickens with the rAAAV-HN. *In ovo* vaccination was implemented mimicking the commercial set up as previously explained (21). Treatments where applied as described: three groups were inoculated *in ovo* with either 10^8 or 10^9 virus molecules of rAAAV-HN in a volume of 0.1 ml or with 0.1 ml of distilled water. On day 1 after hatch, the remaining three groups were inoculated by the intramuscular route with either 10^8 or 10^9 virus molecules of rAAAV-HN in a volume of 0.1 ml or with 0.1 ml of distilled water. Groups were kept in individual isolation units with appropriate husbandry. Ten birds per subgroup were wing bled at 14, 21, 28, 35 and 42 days post inoculation and the serum samples processed using a commercial NDV ELISA test (IDEXX Laboratories Inc., Westbrook, ME). Serum samples obtained at 14, 21 and 28 days form the birds vaccinated intramuscularly with 10^9 virus molecules of the rAAAV-HN, were further evaluated by the hemagglutination inhibition (HI) test performed as described elsewhere (4), by indirect immunoflourescence (IF) antigen detection of NDV infected CEF and by the virus neutralization test (VN) in chicken embryonating eggs.

Recombinant AAAV-HN vaccine/challenge experiment.

In order to evaluate the protection in broilers against a NDV lethal challenge conferred by the rAAAV-HN virions, one hundred and fifteen 1-day old broiler chicken embryos were collected form a commercial hatchery (Maracay, Venezuela). Ten of the embryos were hatched and bled to evaluate maternal antibody titers by the HI test (4). The remaining eggs were divided in seven groups of 15 and incubated separately. The rAAAV-HN dose used for vaccination was 10^9 virus molecules in a volume of 0.1 ml. The first group of birds was vaccinated *in ovo* at the 18^{th} day of incubation; the second group was vaccinated *in ovo* at the day 18^{th} of incubation and revaccinated intramuscularly (IM) at day seven after hatch. The third, forth and fifth groups were vaccinated IM at 1, 1+7 or 7 days of age, respectively. The remaining two groups served as nonvaccinated challenged and nonchallenged controls. Birds were placed in cages at the National Veterinary Research Institute in Maracay, Venezuela where water and commercial feed was provided *ad libitum*. All vaccinated birds and the nonvaccinated/challenged group were inoculated at 28 days of age by oculo-nasal instillation with $10^{3.0}$ EID_{50}/0.1 ml of a Venezuelan velogenic viscerotropic NDV field isolate. All birds were observed for 10 days and morbidity and mortality was recorded.

Statistical analysis: Statistical analysis was performed using the Sigma Stat 3.0 software (Chicago, IL.USA). Antibody levels were analyzed using the Dunn's method and the SNK test performed at $P \leq 0.05$. For the vaccine challenge trial, the influence of treatments on the relative distribution frequency of protection was analysed by Chi-squared tests and by the Fisher's exact test when the number of observation was between 5 and 3, respectively.

RESULTS.

***In vitro* assessment of HN expression from plasmid constructs.** After transfection of HEK 293 monolayers with the pAAAV-NDV-HN plasmids, HN protein expression was recognized by NDV specific antiserum as demonstrated by immunocytochemistry positive staining (Fig 2).

Generation of rAAAV-HN particles. Simultaneous transfection of HEK 293 monolayers with a plasmid containing the HN gene, a plasmid expressing the Rep and Cap coding regions of AAAV and a commercial pHelper plasmid, generated rAAAV-HN virions detected by immunocytochemistry and by electron microscopy (not shown). Viral particles exhibited diameters from 17 to 22 nm.

Size of the expressed HN protein. Western blot analysis of rAAAV-HN infected CEF using a monoclonal NDV HN specific mouse antiserum, showed a 63 KDa protein which was also recognized in CEF infected with LaSota strain, the band was not observed in cells infected with the wild type AAAV (DA-1 strain) or in the uninfected cells (Fig. 3).

Serological evidence of HN protein expression in the host. The ELISA titers at 14, 21, 28, 35 and 42 days of age from the SPF birds vaccinated with the rAAAV-HN are shown in Table 1. Regardless of dose or the route of inoculation used, the ELISA titers of the vaccinated groups differed ($P<0.05$) from the unvaccinated controls at days 35 and 42 of age. The average HI titers at 14, 21 and 28 days of age was 80, 320 and 640, respectively. The antiserum obtained at day 28 from rAAAV-HN vaccinated birds allowed the IF identification of NDV inoculated CEF cells (48 hours post infection), the level of detection was equivalent to that of antiserum raised against live LaSota virus, no signal was obtained in the uninfected controls. In an embryonated chicken egg based neutralization test, a positive virus neutralization index of 2.6 was obtained using rAAAV-HN antiserum.

Vaccine/challenge experiment. Maternal antibody positive broiler chickens with an arithmetic mean HI titer of 120 were used in this trial. The results for the challenge experiment are summarized in Table 2. All the birds in the nonvaccinated–challenged group showed clinical NDV and died within 5 days post-challenge. No clinical signs or mortality were observed in the nonvaccinated-nonchallenged group. The level of protection against mortality conferred by the 10^9 viral particles of rAAAV-HN, ranged in from 0 to 80% in maternal antibody positive broiler chickens vaccinated *in ovo* and/or by the intramuscular route at different time points. The morbidity and mortality levels observed after virulent challenge were lower in all vaccinated groups when compared with the challenged control, with the exception of the group where only one dose of the recombinant virus was applied *in ovo*. The highest level of protection (80%) was detected in the group primed *in ovo* at day 18^{th} of incubation and revaccinated at seven days of age. Groups with two doses of the rAAAV-HN vaccine survived better the challenge than groups where only one dose was applied.

DISCUSSION.

Adeno-associated viruses are being developed as vectors for human gene therapy and based on its lack of pathogenicity and long lasting high levels of transgene expression represent a promising candidate for poultry vaccination (16, 25, 32). Currently adeno-associated virus vector genomes contain the transgene(s) and regulatory sequences between two copies of the virus ITRs, hence no viral genes are expressed and little of the unique sequence of the adeno-associated virus remains to induce anti-vector immunity (16, 33). High levels of long lived transgene expression in adeno-associated virus vectors are related to an adequate behavior of foreign promoters in the context of an AAAV vector (7, 16). In this trial, the appropriate orientation of the HN gene with respect of the CMV promoter in pAAAV-NDV-HN transfected

HEK293 cells was confirmed by immunocytochemistry detection of transgenic HN protein using NDV specific antiserum, indicating that the expressed HN protein structure was appropriate for antigen recognition.

Assembly of rAAAV-HN virions was detected by electron microscopy, the size of the viral particles correspond to the normal size of the AAV assembled capsids (5). The rAAAV-HN expression of a HN protein with the expected molecular weight of 63 kDa was demonstrated by western blot analysis of rAAAV-HN infected CEF. These results agree with our previous *in vitro* observations on the ability of the rAAAV virions to infect and induce adequate protein expression in chicken cells (9). Further confirmation of appropriate HN conformation was obtained by the IF detection of NDV infected CEF using antiserum from rAAAV-HN inoculated chickens, indicating that the bird's immune system can recognize the expressed HN and mount a neutralizing immune response as demonstrated by the high virus neutralization index obtained.

The NDV specific ELISA and HI titers represent quantifiable evidence of transgenic expression of the recombinant HN protein and its suitability to be recognized by the chicken immune system. However, in this trial the ELISA titers were not uniform, delayed and relatively low in levels when compared with responses induced by live NDV vaccinations (1, 2, 23). The variability of the observed immune response could be the consequence of a previously reported tendency of the recombinant adeno-associated virus particles to agglutinate spontaneously, the aggregation of recombinant AAAV seems to be directly associated with variability in levels of empty capsids and DNA or protein impurities in the vector preparations, leading to reduced yield and less efficient gene transfer (20). In addition, the use of a commercial ELISA test with a solid phase coated with complete NDV particles might not be the most sensitive system to detect response elicited by the rAAAV-HN, which only induces the expression of the viral HN proteins.

The later observation seem to be sustained by the earlier antibody detection observed using the HI test in the rAAAV-HN vaccinated birds (response detected as early as 14 days) and in the way that these antibodies correlated with a high neutralizing activity.

Being these initial studies on the use of avian adeno-associated viruses for transgene expression of immunogenic proteins from poultry viruses, a pilot study to test protection was designed as a proof of concept. However, the fact that exotic NDV is select agent imposes strong limitations for challenge studies. In this trial, the challenge was performed overseas where the facilities determined the number of birds used in the experiment. As shown in Table 2, protection against lethal challenge was observed in four out of five rAAAV-HN vaccinated groups these results are similar to the levels of protection reported for other recombinant vaccines against NDV (14, 18, 28).

The birds vaccinated twice with the rAAAV-HN seem to have been primed and showed better protection that those birds vaccinated once (1, 23). It is interesting to notice that in countries where velogenic Newcastle disease is endemic, the broiler vaccination programs include multiple vaccine applications in both the hatchery and field (31). The *in ovo* route is popular due to increased speed, reduced labor costs, and uniform vaccination (21). Overall, the serological and challenge results observed for the birds vaccinated with the rAAAV-HN *in ovo* (up to 80% protection in birds primed *in ovo* and revaccinated at seven days), represent an indication of the usefulness of the rAAAV-HN as a vector vaccine for initial vaccination by the *in ovo* route when field revaccinations are scheduled.

An additional advantage of this recombinant system is the possibility of using HN proteins homologous to those of the field strains. Although all the strains of NDV are considered to be within the same serotype, increasing the genetic relatedness of the NDV vaccine virus to the

challenge virus can produce more specific neutralizing antibodies, it has been demonstrated that HI titers are higher when the HN antigen used in the HI assay is homologous with the antigen used to prepare vaccines (26). Recently, Miller et al.(15) demonstrated that vaccines homologous with the challenge virus reduced oral shedding significantly more than the heterologous vaccines. Hence, NDV vaccines formulated to be phylogenetically closer to potential outbreak viruses may provide better ND control by reducing virus transmission from infected birds. Similar observations have been reported for avian influenza with reduction of oropharyngeal viral shed in vaccinated and infected birds (27).

In summary, the generation of rAAAV particles expressing the HN protein was demonstrated by immunocytochemistry, electron microscopy and western blot analysis, serological evidence of *in vivo* expression was obtained in SPF birds (ELISA, HI, IF, VN) and protection against virulent challenge was observed in broilers. Hence, the feasibility of developing and using an avian adeno-associated virus based gene delivery system that represents a new vaccine candidate for NDV was demonstrated.

Acknowledgements.

The authors will like to thank to Dr. Egbert Mundt and Mark Jackwood from the University of Georgia for their help and valuable suggestions.

REFERENCES

1. Al-Garib, S. O., A. L. J. Gielkens, E. Gruys, and G. Koch. Review of Newcastle disease virus with particular references to immunity and vaccination. Worlds Poultry Science Journal 59:185-200. 2003.

2. Alexander, D. J. Newcastle disease and other avian paramyxoviruses. Rev Sci Tech 19:443-462. 2000.

3. Alexander, D. J. Gordon Memorial Lecture. Newcastle disease. Br Poult Sci 42:5-22. 2001.

4. Allan, W. H., and R. E. Gough. A standard haemagglutination inhibition test for Newcastle disease. (1). A comparison of macro and micro methods. Vet Rec 95:120-123. 1974.

5. Berns, K. I., and S. Adler. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. J Virol 9:394-396. 1972.

6. Bossis, I., and J. A. Chiorini. Cloning of an avian adeno-associated virus (AAAV) and generation of recombinant AAAV particles. J Virol 77:6799-6810. 2003.

7. Chiorini, J. A., L. Yang, Y. Liu, B. Safer, and R. M. Kotin. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J Virol 71:6823-6833. 1997.

8. Estevez, C., and P. Villegas. Sequence analysis, viral rescue from infectious clones and generation of recombinant virions of the avian adeno-associated virus. Virus Res 105:195-208. 2004.

9. Estevez, C., and P. Villegas. Recombinant avian adeno-associated virus: transgene expression in vivo and enhancement of expression in vitro. Avian Dis 50:216-221. 2006.

10. Ferreira, T. B., P. M. Alves, J. G. Aunins, and M. J. Carrondo. Use of adenoviral vectors as veterinary vaccines. Gene Ther 12 Suppl 1:S73-83. 2005.

11. Francois, A., N. Eterradossi, B. Delmas, V. Payet, and P. Langlois. Construction of avian adenovirus CELO recombinants in cosmids. J Virol 75:5288-5301. 2001.

12. Gravel, K. A., and T. G. Morrison. Interacting domains of the HN and F proteins of newcastle disease virus. J Virol 77:11040-11049. 2003.

13. Mayo, M. A. Virus taxonomy - Houston 2002. Arch Virol 147:1071-1076. 2002.

14. Meulemans, G., C. Letellier, M. Gonze, M. C. Carlier, and A. Burny. Newcastle-Disease Virus-F Glycoprotein Expressed from a Recombinant Vaccinia Virus Vector Protects Chickens against Live-Virus Challenge. Avian Pathology 17:821-827. 1988.

15. Miller, P. J., D. J. King, C. L. Afonso, and D. L. Suarez. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 25:7238-7246. 2007.

16. Muzyczka, N. a. K. B. Parvoviridae: the viruses and their replication. In: Fields Virology. D. a. H. P. E. Knipe, ed. Lippincott-Raven., Philadelphia. pp 2327-2379. 2001.

17. Nagy, E., P. Huber, P. J. Krell, and J. B. Derbyshire. Synthesis of Newcastle disease virus (NDV)-like envelopes in insect cells infected with a recombinant baculovirus expressing the haemagglutinin-neuraminidase of NDV. J Gen Virol 72 (Pt 3):753-756. 1991.

18. Nagy, E., P. J. Krell, R. A. Heckert, and J. B. Derbyshire. Vaccination of chickens with a recombinant fowlpox virus containing the hemagglutinin-neuraminidase gene of Newcastle disease virus under the control of the fowlpox virus thymidine kinase promoter. Can J Vet Res 58:306-308. 1994.

19. Nishino, Y., M. Niikura, T. Suwa, M. Onuma, B. Gotoh, Y. Nagai, and T. Mikami. Analysis of the protective effect of the haemagglutinin-neuraminidase protein in Newcastle disease virus infection. J Gen Virol 72 (Pt 5):1187-1190. 1991.

20. Qu, G., C. Connolly, A. Koblansky, J. M. Sommer, A. McClelland, and J. F. Wright. Evidence that ionic interactions are involved in concentration-induced aggregation of recombinant adeno-associated virus. Molecular Therapy 7:S348-S348. 2003.

21. Ricks, C. A., A. Avakian, T. Bryan, R. Gildersleeve, E. Haddad, R. Ilich, S. King, L. Murray, P. Phelps, R. Poston, C. Whitfill, and C. Williams. In ovo vaccination technology. Adv Vet Med 41:495-515. 1999.

22. Scheid, A., and P. W. Choppin. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology 57:475-490. 1974.

23. Senne, D. A., D. J. King, and D. R. Kapczynski. Control of Newcastle disease by vaccination. Dev Biol (Basel) 119:165-170. 2004.

24. Snyder, R. O. Adeno-associated virus-mediated gene delivery. J Gene Med 1:166-175. 1999.

25. Snyder, R. O., and J. Francis. Adeno-associated viral vectors for clinical gene transfer studies. Curr Gene Ther 5:311-321. 2005.

26. Stone, H. D. Efficacy of oil-emulsion vaccines prepared with pigeon paramyxovirus-1, Ulster, and La Sota Newcastle disease viruses. Avian Dis 33:157-162. 1989.

27. Taylor, J., R. Weinberg, Y. Kawaoka, R. G. Webster, and E. Paoletti. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 6:504-508. 1988.

28. Taylor, J., L. Christensen, R. Gettig, J. Goebel, J. F. Bouquet, T. R. Mickle, and E. Paoletti. Efficacy of a recombinant fowl pox-based Newcastle disease virus vaccine candidate against velogenic and respiratory challenge. Avian Dis 40:173-180. 1996.

29. Timms, L., and D. J. Alexander. Cell-Mediated Immune-Response of Chickens to Newcastle-Disease Vaccines. Avian Pathology 6:51-59. 1977.

30. Tsurudome, M., M. Kawano, T. Yuasa, N. Tabata, M. Nishio, H. Komada, and Y. Ito. Identification of regions on the hemagglutinin-neuraminidase protein of human parainfluenza virus type 2 important for promoting cell fusion. Virology 213:190-203. 1995.

31. Villegas, P. Viral diseases of the respiratory system. Poult Sci 77:1143-1145. 1998.

32. Wright, J. F., G. Qu, C. Tang, and J. M. Sommer. Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. Curr Opin Drug Discov Devel 6:174-178. 2003.

33. Zaiss, A. K., and D. A. Muruve. Immune responses to adeno-associated virus vectors. Curr Gene Ther 5:323-331. 2005.

Table 3.1. ELISA antibody titers against NDV after different doses and routes of application for the rAAAV-HN product in SPF chickens.

Route	Inoculum	Virus particles/0.1ml	Sampling age (days) [A]				
			14	**21**	**28**	**35**	**42** [B]
In ovo 18th day-incubation	rAAAV-HN	10^9	0	0	0	1076 [a]	3990 [a]
		10^8	0	0	0	520 [b]	1960 [b]
	PBS [C]	-	0	0	0	0 [c]	0 [c]
Intramuscular in breast.	rAAAV-HN	10^9	0	543	847	1072 [a]	1905 [b]
		10^8	0	0	0	729 [b]	2177 [b]
	Distilled water	-	0	0	0	0 [c]	0 [c]

[A] Geometric mean titer of ten birds per subgroup.
[B] At days 35 and 42 P.I the means with the same letter within column are not significantly different by the SNK test ($P < 0.05$).
[C] Phosphate buffer saline

Table 3.2. The rAAAV-HN vaccine challenge experiment in broilers.

Route/age of vaccination	Morbidity[A]	Mortality	Protection	%
In ovo day 18	15/15	15/15	0/15	0
In ovo day 18 + IM day 7	5/15	3/15	12/15	80 *
Intramuscular day 1	8/15	7/15	8/15	54
Intramuscular day 1+7	8/15	5/15	10/15	67
Intramuscular day 7	10/15	8/15	7/15	47 *
Unvaccinated/challenged	15/15	15/15	0/15	0
Unvaccinated /unchallenged.	0/15	0/15	15/15	100

[A] All vaccinated birds and the nonvaccinated/challenged group were inoculated at 28 days of age by oculo-nasal instillation with $10^{3.0}$ EID_{50}/0.1 ml of a Venezuelan velogenic viscerotropic NDV field isolate. All birds were observed for 10 days and morbidity and mortality was recorded.
* The level of protection was compared, the 80% protection level was significantly (P=0.05) higher than the 47% protection level.

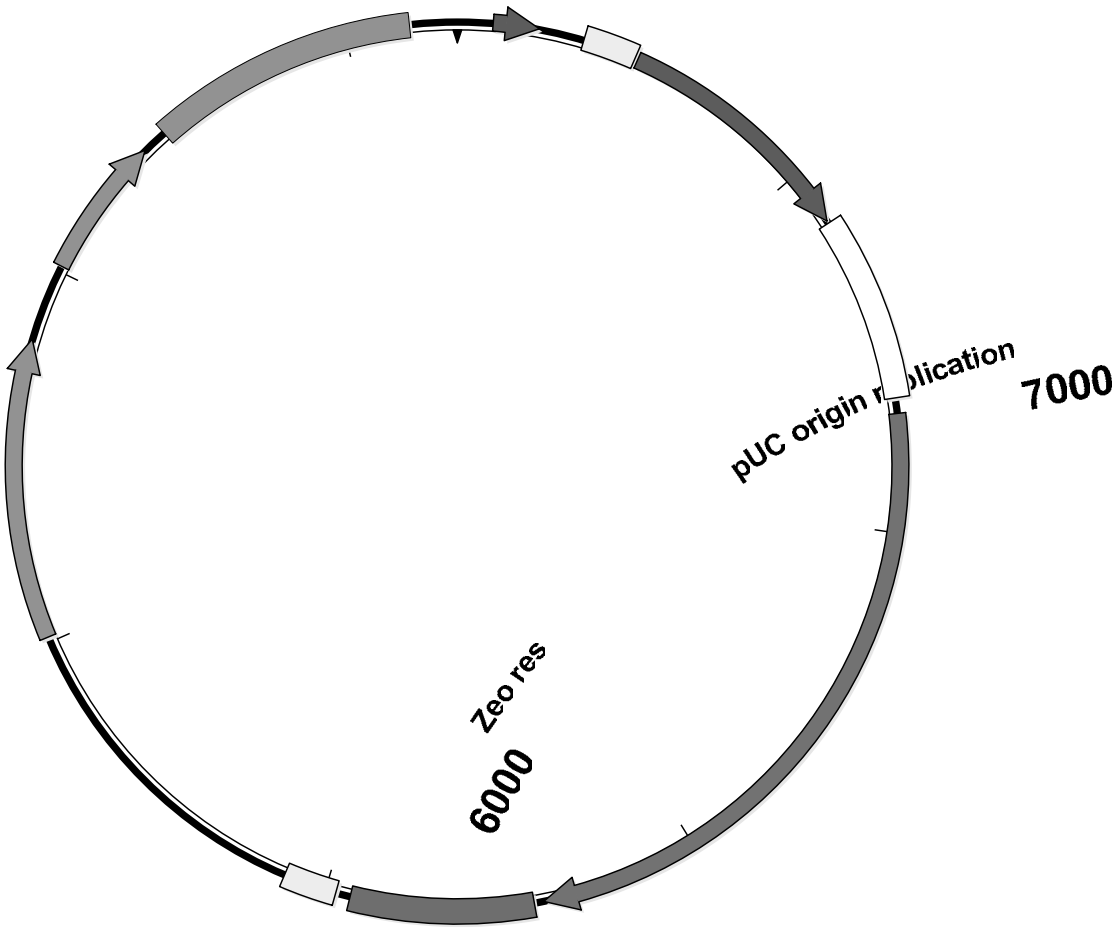


Fig. 3.1 pAAAV-NDV-HN plasmid construct. DNA fragments of 1341 bp (HN gene) recovered were cloned downstream of the late cytomegalovirus (CMV) promoter in a plasmid construct containing the inverted terminal repeats (ITR) of the DA-1 strain of AAAV.

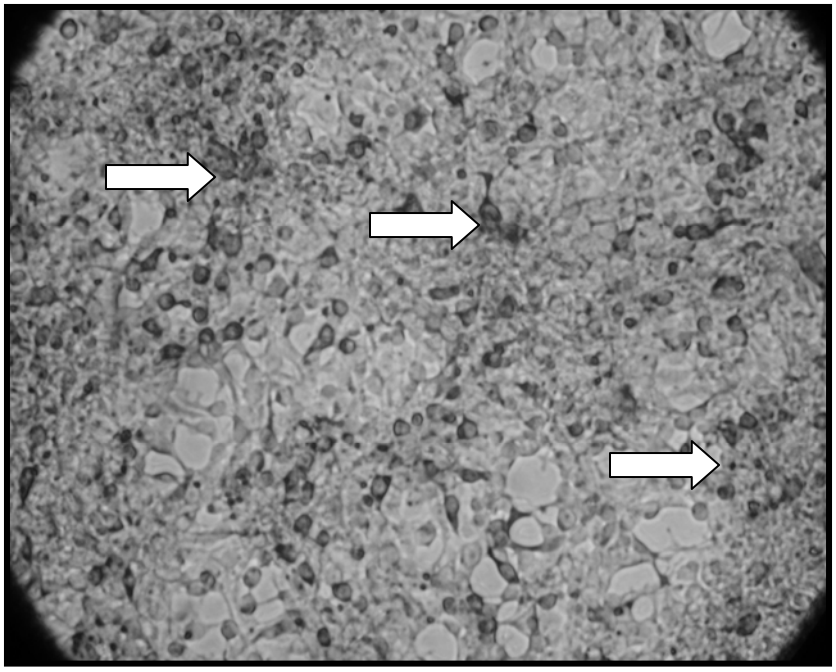


Fig. 3.2 Immunocytochemistry detection of HN protein expression. The expression of the HN proteins was detected on pAAAV-NDV-HN transfected cells by immunocytochemistry. The presence of positive dark staining (arrows) in the transfected HEK 293 cells was considered positive for HN expression.

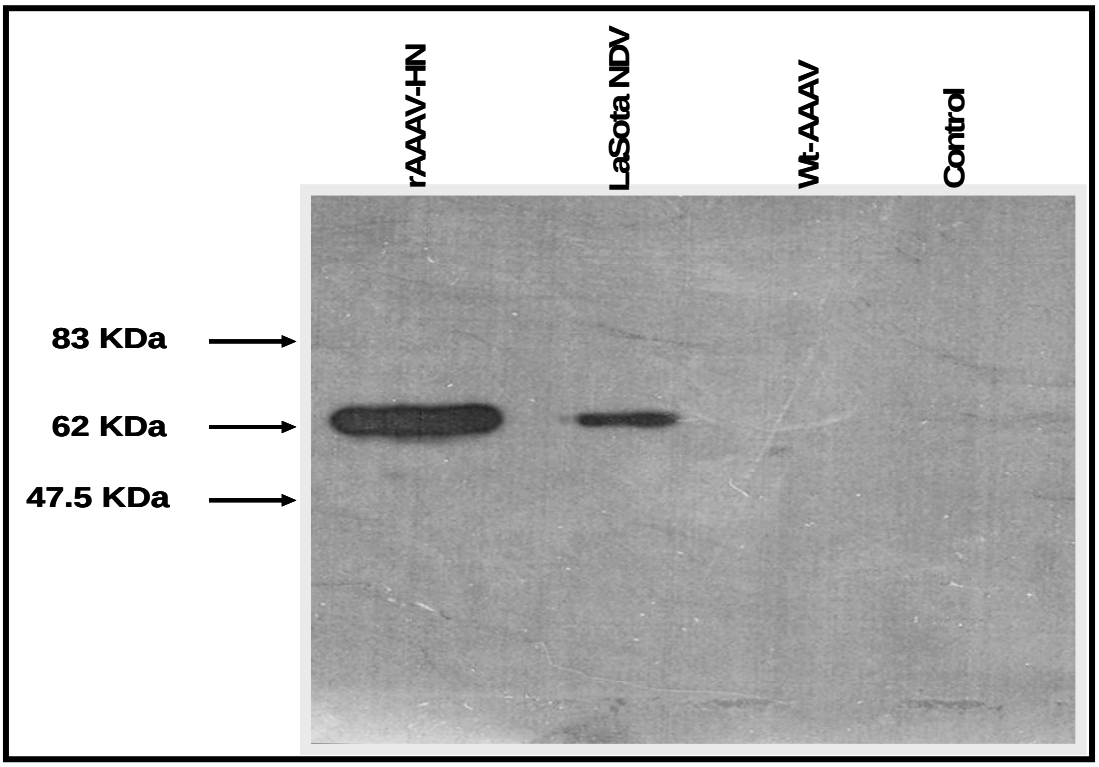


Fig. 3.3 Western blot analysis of infected CEF. Cells infected with the rAAAV-HN, LaSota NDV strain, wild type AAAV and negative control. Anti-HN monoclonal antiserum was used to detect a 63 kDa protein in the rAAAV-HN and LaSota infected cells, no signal was observed in the wild type AAAV or uninfected cells.

CHAPTER IV

A RECOMBINANT AVIAN ADENO-ASSOCIATED VIRUS AS A VECTOR FOR INFECTIOUS BURSAL DISEASE VACCINATION [1].

---

[1] F. Perozo, P. Villegas, C. Estevez, I. R. Alvarado, L. B. Purvis. **Revista Cientifica FCV-LUZ. VOL XVII, 1-6. 2007.**
Reprinted here with permission of publisher.2/3/2008

SUMMARY.

Infectious bursal disease is a worldwide distributed immunosuppressive disease of young chickens that need to be controlled by vaccination; it represents one of the main concerns for the poultry industry. The adeno-associated viruses are non-pathogenic viruses, capable of accommodating relatively long pieces of DNA, and of infecting a wide variety of cell types. A member of this family, the avian adeno-associated virus, has been fully characterized and successfully used for gene delivery in chicken embryo tissues and cells. In this study it was demonstrated by electron microscopy and immunocitochemistry the feasibility of generating recombinant avian adeno-associated virus (rAAAV) virions expressing the immunogenic viral protein 2 of infectious bursal disease virus (IBDV). Serological evidence of VP2 protein expression measured as IBDV specific antibody response after *in ovo* or intramuscular inoculation of the recombinant virus in specific pathogen free (SPF) chickens was observed. The use of rAAAV virions for gene delivery in poultry is a promising approach to poultry vaccination.

Key words: Avian adeno-associated virus, vaccination, immune response, infectious bursa disease.

RESUMEN

El virus de la enfermedad infecciosa de la bolsa es una enfermedad de distribución mundial que afecta a aves jóvenes que debe ser controlada mediante vacunación y constituye una de las preocupaciones principales de la industria avícola mundial. Los virus adeno-asociados aviares son virus no patogénicos capaces de dar cabida a porciones relativamente largas de ADN y de infectar una amplia variedad de tipos celulares. Un miembro de esta familia, el virus adeno-asociado aviar ha sido caracterizado por completo y utilizado como un vector para la entrega de

genes en células y tejidos de embriones de pollo. En el presente estudio se demostró mediante inmunohistoquímica y microscopia electrónica la factibilidad de generar virus adeno-asociados recombinantes expresando la proteína viral 2 del virus de la enfermedad infecciosa de la bolsa. Luego de la inoculación *in ovo* o intramuscular de aves libres de patógenos específicos con el virus recombinante, se observó evidencia serológica de la expresión de la proteína VP2. La utilización de virus adeno-asociado aviar recombinantes para la entrega de genes es una opción interesante para la vacunación de aves domesticas.

Palabras clave: Virus adeno-asociado aviar recombinante, vacunacion, respuesta inmune, enfermedad infecciosa de la bolsa.

INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family, the genome of which consists of two segments (A and B) of double-stranded RNA [14]. Segment A encodes a polyprotein that can be cleaved by auto proteolysis to form mature viral proteins VP2, VP3 and VP4 [12]. The antigenic site responsible for the induction of neutralizing antibodies is highly conformation-dependent and located on the VP2 [4]. IBDV multiplies rapidly in developing B lymphocytes in the bursa of Fabricius, and causes infectious bursal disease (IBD) in chickens inducing clinical signs, immunosuppression, and can act as a predisposing factor for the development of several other diseases [1]. Control of IBD in young chickens have been primarily achieved by vaccination with live attenuated strains of IBDV early in life, as well as by transferring high levels of maternal antibody induced by the administration of live and killed IBD vaccines to the breeders [13].

Advances in the understanding of viral diseases pathogenesis and of the molecular mechanisms involved in the generation of protective immune responses, have opened new avenues for the prevention of these diseases. The use of recombinant viruses for gene delivery as a vaccination strategy in veterinary medicine has been previously documented [9]. Immunization of chickens against IBD using a vector that *in vivo* expresses VP2 has been previously reported using an avian herpesvirus vector [20], a fowlpox virus vector [17], a fowl adenovirus vector [10] and a Marek's disease virus vector [19].

In recent years, an extensive amount of work has been performed for the characterization and use of replication defective parvoviruses for the purpose of gene delivery [9]. These replication defective parvoviruses, the adeno-associated viruses (AAV), are non-pathogenic, capable of accommodating relatively long pieces of DNA, and capable of infecting a wide variety of cell types [18]. A member of this family, the avian adeno-associated virus (AAAV) has been fully characterized [3, 8] and successfully used for gene delivery in chicken embryo tissues and cells [7, 8]. The aim of this work was to generate recombinant AAAV virions (rAAAV) expressing the immunogenic VP2 peptides of the Edgar strain of IBDV and assess their ability to generate immunity in chickens.

MATERIALS AND METHODS.

Amplification of the VP2 genes by reverse transcriptase-polymerase chain reaction (RT-PCR).

Amplification of the VP2 gene of the Edgar strain of IBDV (1734 bp) was performed using the forward VP2 (5'-AGCATCGATATGGACCGCGCCGTTA-3') and reverse VP2 (5'-AGCAGATCTTTACTAGCCAGACCTGGCTTCTCTA-3') primers. The *Cla*I and *Bgl*II

restriction sites were included in the 5' end of the forward and reverse primers, respectively. Reverse transcription (RT) was performed with the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA. USA) while polymerase chain reactions (PCR) were carried out with the FailSafe PCR system and PCR 2X premix C (Epicentre, Madison, WI. USA.) following manufacturers' instructions. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml). The products of the proper size were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA. USA) using manufacturers' recommendations.

Cloning and sequencing of the VP2 genes.

The amplified VP2 genes were cloned into pCR 2.1 plasmids (Invitrogen, Carlsbad, CA) and transformed into TOP 10 *E. coli* cells following manufactures' recommendations. Positive colonies were selected and recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA. USA). The presence of the VP2 genes was verified by digestion of these plasmids with the *Cla*I and *Bgl*II restriction enzymes. Electrophortic analysis was performed as explained. In order to confirm the appropriated sequence, direct nucleotide sequencing of the VP2 gene was performed using the respective forward and reverse primers with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA. USA).

Generation of plasmid constructs to produce rAAAV particles coding for the VP2 genes.

DNA fragments of 1734 bp (VP2 gene) recovered from the pCR 2.1 plasmid after restriction enzyme digestion were cloned in a plasmid construct named p3.6 ITR-MCS containing the internal terminal repeats (ITR) of the AAAV [8]. Briefly, the p3.6 ITR-MCS plasmid was linearized with the *Cla*I and *Bgl*II restriction enzymes and dephosphorylated with

calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Inc., Beverly, MA. USA) at 37 C x 30 min. Equimolar concentrations of the VP2 genes were ligated using T4 DNA ligase and transfected using TOP 10 *E. coli* cells following manufactures' instructions. Positive transformed colonies were selected, the presence of the VP2 genes in the p3.6 ITR-MCS was verified after digestion with the *Cla*I and *Bgl*II restriction enzymes by electrophoresis analysis as previously mentioned.

Expression of the VP2 proteins.

Human embryo kidney cells expressing the E1A and E1B immediate early genes of human adenovirus type 5 (HEK 293) were obtained from ATCC (CRL-1573) and grown using Dulbecco's modified minimal essential medium (Gibco, Carlsbad, CA. USA) supplemented with 10% fetal bovine serum and antibiotics in 25 cm^2 cell culture flasks and subcultured in 35 mm^2 plates until 90% confluence. Ten micrograms of the p3.6 ITR-MCS plasmid containing the VP2 genes were transfected with the use of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA. USA), as recommended by the manufacturer. The expression of the VP2 proteins was detected 72 hr post-transfection by immunohistochemistry (IHC) using the Diaminobenzidine-Urea-Peroxidase complex (Sigma Chemicals Co., St Louis, MO. USA) following manufacturer's instructions. Commercial polyclonal antibodies against IBDV Edgar strain (SPAFAS Inc., Norwich, CT. USA) were used in the test. The presence of positive dark brown staining in the transfected HEK 293 cells was considered as positive protein expression.

Generation of rAAAV virions expressing the VP2 proteins.

Recombinant AAAV virions were generated by simultaneous transfection of HEK 293 cell monolayers, with three plasmid constructs: the p3.6 ITR-MCS containing the VP2, the pRC-CMV expressing the Rep and Cap coding regions of AAAV [8] and a commercial pHelper

plasmid coding for the E2, E4 and VR-RNA genes derived from the human adenovirus type 5 (Stratagene, La Jolla, CA. USA). The purification of the recombinant viral particles was accomplished 48 hr post-transfection using ultracentrifugation on cesium chloride cushion and density gradients [5]. The presence of the rAAAV virions coding for the VP2 gene (rAAAV-VP2) was confirmed by electron microscopy following standard procedures.

Systemic immune response against the rAAAV.

One hundred and twenty 18 day-old specific pathogen free (SPF) embryos (Sunrise Farms. Catskil. NY) were divided in 6 groups of 20 embryos and used to test the serological response to two different doses (10^8 or 10^9 virus molecules/ml) and two administration routes (*in ovo* or intramuscular inoculation of 0.1 ml) for the of the rAAAV-VP2 as described in Table 1 of the results section. Once hatched, birds were kept in BSL2 isolation units at the PDRC where appropriated husbandry was provided. Ten birds in each subgroup were wing bled at 14, 21, 28, 35 and 42 days of age and the serum was refrigerated until processed for the presence immunoglobulin G (IgG) against IBDV using a commercial ELISA test (IDEXX Laboratories Inc., Westbrook, ME. USA) following manufacturer's protocols.

Statistical analysis:

All statistical analysis was performed using the Sigma Stat 3.0 software (1- way ANOVA, multiple comparison tests). SNK test was performed at $P \leq 0.05$.

RESULTS AND DISCUSSION.

The appropriate orientation of the VP2 gene with respect of the major late cytomegalovirus promoter was confirmed by immunohistochemistry as demonstrated by positive recognition of p3.6 ITR-MCS / VP2 transfected cells by IBDV specific antiserum. No staining

was observed in non transfected HEK293 cells proving that the foreign gene induced the expression of the IBDV protein (FIG. 1). In the case of the VP2 protein, a correct structural conformation seems to be very critical due to the conformational dependence of the immunogenic epitopes formed by the close proximity of the hypervariable regions A and B [6].

Forty eight hours after transfection of HEK 293 monolayers with the p3.6 ITR-MCS containing the VP2, the pRC-CMV expressing the Rep and Cap coding regions of AAAV and a commercial pHelper plasmid coding for the E2, E4 and VR-RNA genes, assembly of rAAAV-VP2 virions was demonstrated by electron microscopy with the presence of viral particles exhibiting diameters from 17 to 22 nm (FIG 2), which correspond to the normal size of the AAV assembled capsids [2, 5].

A humoral immune response measured as the geometric mean titer (GMT) of the antibodies against IBD was detected after the inoculation of the rAAAV-VP2 virions in SPF chickens, the results are shown in TABLE 1. Statistically significant differences were observed 42 days of age between all the vaccinated groups and the mock inoculated controls, regardless of the dose or route of inoculation. Seroconversion against IBDV was observed as early as 14 days after inoculation of the rAAAV-VP2. The highest antibody titers (GMT of 3997) corresponded to the *in ovo* inoculated group using 10^8 genomic copies/ml at day 42. When applied by the intramuscular route the rAAAV-VP2 elicited a more consistent antibody response along the different time points tested. This is the first attempt to use a rAAAV for vaccination against poultry pathogens, previous work demonstrated that the recombinant virus was able to induce the production of a reporter gene in tissues derived from the rAAAV inoculated embryos [7], the presence of a measurable immune response confirms the ability of the rAAAV virions for gene delivery in poultry.

Even tough the presence of systemic humoral immune response measured as IgG titers against IBDV in the inoculated SPF birds was detected, the response was not uniform and relatively low in levels. The working mechanism of the gene delivery system used and the pathways for antigen presentation and recognition are not fully understood for chickens [9]. The variability in the results might be explained by a previously reported tendency of recombinant particles to agglutinate spontaneously leading to reduced yield and less efficient gene transfer [11, 15, 21]. Cell mediated immunity is known to be involved in protection against re-infection with IBDV [16]. In this pilot study, the effect of the rAAAV on the local level of protection was not evaluated. Further research including vaccine-challenge trials need to be conducted to assess the whether the rAAAV are able to elicit full protection against IBDV. The use of rAAAV virions for gene delivery in poultry represents a new and promising approach to poultry vaccination.

CONCLUSIONS

The feasibility of generating recombinant avian adeno-associated virions expressing the immunogenic viral protein 2 (VP2) of IBDV was demonstrated.

A detectable systemic immune response measured as antibodies against IBDV was elicited in SPF birds after inoculation with the recombinant virus.

Further research is required to determine the level of protection conferred by the rAAAV-VP2 virions against virulent IBDV and to compare the effects the replication defective recombinant product on the bursa of Fabricius with those of commercial vaccines known to induce bursal damage during viral replication.

REFERENCES

[1]. BECHT, H. Infectious bursal disease virus. **Curr. Top. Microbiol. Immunol.** 90:107-121. 1980.

[2]. BERNS, K.; ADLER, S. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. **J. Virol.** 9:394-396. 1972.

[3]. BOSSIS, I.; CHIORINI, A. Cloning of an avian adeno-associated virus (AAAV) and generation of recombinant AAAV particles. **J. Virol.** 77:6799-6810. 2003.

[4]. CASTON, J.; MARTINEZ-TORRECUADRADA, J.; MARAVER, A.; LOMBARDO, E.; RODRIGUEZ, E.; CASAL, J.; CARRASCOSA, J. C terminus of infectious bursal disease virus major capsid protein VP2 is involved in definition of the T number for capsid assembly. **J. Virol.** 75:10815-10828. 2001.

[5]. CHIORINI, J.; YANG, A.; LIU, Y.; SAFER, B.; KOTIN, R. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. **J. Virol.** 71:6823-6833. 1997.

[6]. COULIBALY, F.; CHEVALIER, C.; GUTSCHE, I.; POUS, J.; NAVAZA, J.; BRESSANELLI, S.; DELMAS, B.; REY, A. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. **Cell** 120:761-772. 2005.

[7]. ESTEVEZ, C.; VILLEGAS, P. Recombinant Avian Adeno-associated Virus: transgene expression *in vivo* and enhancement of expression *in vitro*. **Avian Dis**. 50:216-21. 2006.

[8]. ESTEVEZ, C.; VILLEGAS, P. Sequence analysis, viral rescue from infectious clones and generation of recombinant virions of the avian adeno-associated virus. **Virus. Res**. 105:195-208. 2004.

[9]. FERREIRA, T.; ALVES, P.; AUNINS, J.; CARRONDO, M. Use of adenoviral vectors as veterinary vaccines. **Gene Therapy** 12:73-83. 2005.

[10]. FRANCOIS, A., ETERRADOSSI, N.; DELMAS, B.; PAYET, V.; LANGLOIS, P. Construction of avian adenovirus CELO recombinants in cosmids. **J. Virol**. 75:5288-5301. 2001.

[11]. HUANG, J.; GAO, G.; CROYLE, M.; KURTZMAN, G.; CONCEICAO, C. Aggregation of AAV vectors, its impact on liver-directed gene transfer and development of vector formulations to prevent and dissolve aggregation and enhance gene transfer efficiency. **Mol. Therapy**. 1:286-291. 2000.

[12]. MULLER, H.; BECHT, H. Target cells of infectious bursal disease virus (IBDV) of chickens. **Micro. Infec. Dis. Vir. Parasit.** 251:447-447. 1982.

[13]. MULLER, H.; ISLAM, H.; RAUE, R. Research on infectious bursal disease - the past, the present and the future. **Vet. Micro**. 97:153-165. 2003.

[14]. MULLER, H.; SCHOLTISSEK, C.; BECHT, H. The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. **J. Virol.** 31:584-589. 1979.

[15] QU, G.; CONNOLLY, C.; KOBLANSKY, A.; SOMMER, J.; MCCLELLAND, A.; WRIGHT, A. Evidence that ionic interactions are involved in concentration-induced aggregation of recombinant adeno-associated virus. **Mol. Therapy**. 7:348-356. 2003.

[16]. RAUTENSCHLEIN, S; YEH, H; SHARMA, J. The role of T cells in protection by an inactivated infectious bursal disease virus vaccine. **Vet. Immunol. Immunopathol.** 89:159-67. 2002.

[17]. SHAW, I.; DAVISON, T. Protection from IBDV-induced bursal damage by a recombinant fowlpox vaccine, fpIBD1, is dependent on the titer of challenge virus and chicken genotype. **Vaccine.** 18:3230-3241. 2000.

[18]. SNYDER, R. Adeno-associated virus-mediated gene delivery. **J. Gene. Med**. 1 :166-175. 1999.

[19]. TSUKAMOTO, K.; KOJIMA, C., KOMORI, Y.; TANIMURA, N.; MASE, M.; YAMAGUCHI, S. Protection of chickens against very virulent infectious bursal disease virus (IBDV) and Marek's disease virus (MDV) with a recombinant MDV expressing IBDV VP2. **Virol.** 257:352-362. 1999.

[20]. TSUKAMOTO, K.; SAITO, S.; SAEKI, S.; SATO, T.; TANIMURA, N.; ISOBE, T.; MASE, M.; IMADA, T.; YUASA, N.; YAMAGUCHI, S. Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. **J. Virol.** 76:5637-5645. 2002.

[21].WRIGHT, J.; QU, G.; TANG, C.; SOMMER, J. Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. **Curr. Opin. Drug Discov. Devel**. 6(3):174-178. 2003.

TABLE 4.1.
ANTIBODY TITERS AGAINST IBDV AFTER VACCINATION WITH DIFFERENT DOSES AND ROUTES OF APPLICATION OF rAAAV-VP2./ TITULOS DE ANTICUERPOS CONTRA IBDV POSTERIOR A LA VACUNACION CON DIFERENTES DOSIS DE rAAAV -VP2 Y DIFERENTES RUTAS DE APLICACIÓN.

Route	Inoculum	Dose (genomic copies/ml)	Sampling age (days)*				
			14**	**21**	**28**	**35**	**42**
In ovo	rAAAV-VP2	10^9	0 [b]	1095 [b]	0 [b]	0 [c]	3997 [a]
		10^8	0 [b]	0 [c]	0 [b]	0 [c]	1914 [b]
	control	-	0 [b]	0 [c]	0 [b]	0 [c]	0
Intramuscular	rAAAV-VP2	10^9	0 [b]	2177 [a]	0 [b]	581 [b]	1034 [c]
		10^8	304 2[a]	0 [c]	1469[a]	1331 [a]	2413 [b]
	control	-	0	0 [c]	0 [b]	0 [c]	0 [d]

*Geometric mean titer of ten birds per subgroup.
** Means with the same letter within column are not significantly different by the SNK test (P <0.05)

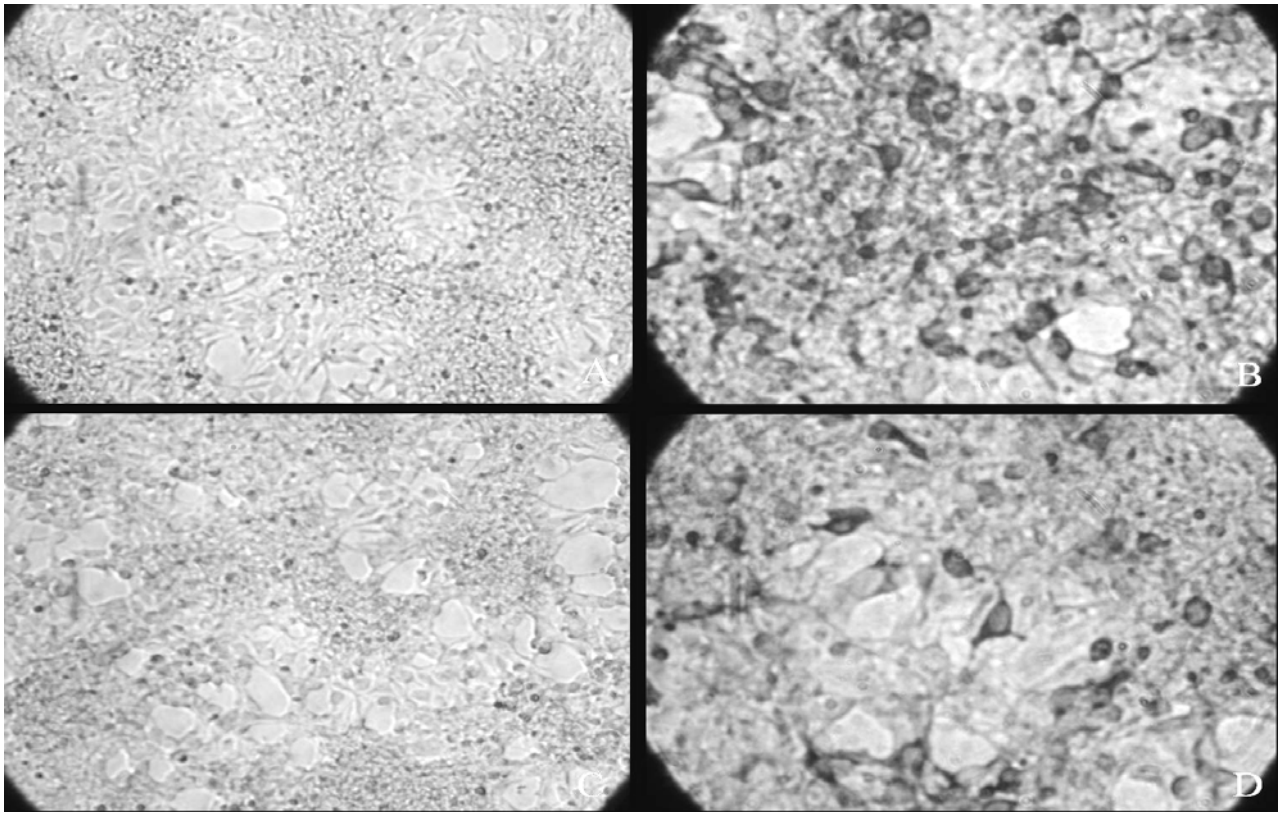


FIGURE 4.1. VP2 PROTEIN EXPRESSION 72 HR AFTER TRANSFECTION (B AND D). NO STAINING WAS OBSERVED IN THE NON-TRANSFECTED HEK 293 MONOLAYERS (A & C) / EXPRESION DE LA PROTEINA VP2 72 HORAS DESPUES DE LA TRANSFECCION (B y D). NO SE OBSERVÓ TINCION EN LAS CELULAS DE EMBRIONARIAS DE RIÑON HUMANO NO TRANSFECTADAS.

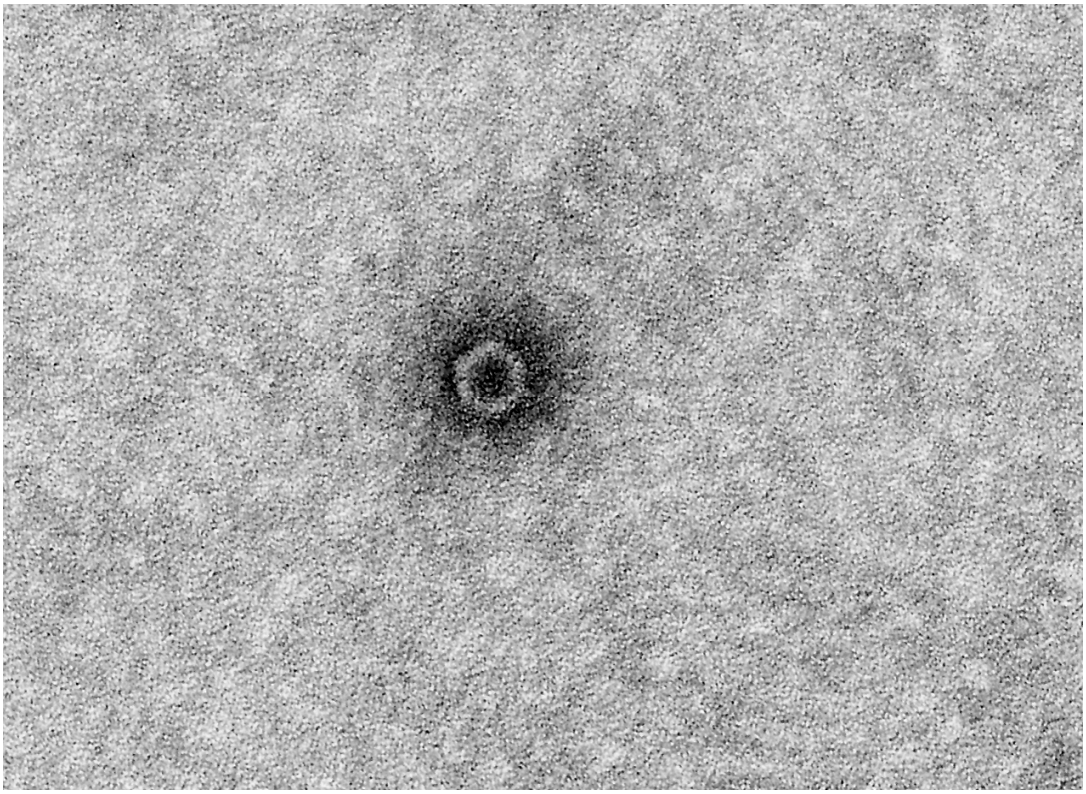


FIGURE 4.2. DETECTION OF RECOMBINANT AAAV VIRIONS BY ELECTRON MICROSCOPY. / DETECCION DE PARTICULAS RECOMBINANTES DE AAAV MEDIANTE MICROSCOPIA ELECTRONICA.

CHAPTER V

PROTECTION AGAINST INFECTIOUS BURSAL DISEASE VIRULENT CHALLENGE CONFERRED BY A RECOMBINANT AVIAN ADENO-ASSOCIATED VIRUS VACCINE [1].

---

[1] F. Perozo, P. Villegas, C. Estevez, I. R. Alvarado, L. B. Purvis, S. Williams. **Accepted by Avian Diseases.**
Reprinted here with permission of the publisher**.**03/03/2008

SUMMARY.
The development and use of recombinant vaccine vectors for the expression of poultry pathogens proteins is an active research field. The adeno-associated virus (AAV) is a replication defective virus member of the family *Parvoviridae* that has been successfully used for gene delivery in humans and other species. In this experiment, an avian adeno-associated virus (AAAV) expressing the infectious bursal disease virus (IBDV) VP2 protein (rAAAV-VP2) was evaluated for protection against IBDV virulent challenge. Specific pathogen free (SPF) birds were inoculated with the rAAAV-VP2 or with a commercial intermediate IBDV vaccine and then challenged with the Edgar strain. IBDV specific antibody levels were observed in all vaccinated groups, titers were higher for the commercial vaccine group. Live commercial vaccine induced adequate protection against morbidity and mortality, nevertheless initial lymphoid depletion and follicular atrophy related to active viral replication was observed as early as day 14 persisting up to day 28 when birds were challenged. No bursal tissue damage due to rAAAV-VP2 vaccination was observed. Eight out of ten rAAAV-VP2 vaccinated birds survived the challenge and showed no clinical signs. The bursa/body weight ratio and bursa lesion scores in the rAAAV-VP2 group indicated protection against challenge. Therefore, transgenic expression of the VP2 protein after rAAAV-VP2 vaccination induced protective immunity against IBDV challenge in 80% of the birds, without compromising the bursa of Fabricius. The use of rAAAV virions for gene delivery represents a novel approach to poultry vaccination.

Key words: Avian adeno-associated virus, vaccination, protection, infectious bursa disease.

INTRODUCTION

Infectious bursal disease virus (IBDV) is the etiological agent of the worldwide distributed infectious bursal disease, also known as Gumboro disease. The IBDV destroys the B lymphocyte precursors found within the bursa of Fabricius inducing bursal atrophy, mortality and/or

immunosuppression in unprotected flocks (3). Very virulent IBDV strains emerged in Europe in the late eighties, they can cause up to 60% mortality and are now considered a threat in several countries worldwide (1, 7). The IBDV belongs to the family *Birnaviridae* (genus *Avibirnavirus*) which includes viruses with bisegmented dsRNA genomes (13). The IBDV genome is divided in segments A and B, segment A has 2 overlapping open reading frames (ORF), the first ORF of segment A encodes the non-structural protein VP5, probably involved in virus release and viral pathogenesis (9). The other portion of segment A encodes a precursor polyprotein in a large ORF, the product of which is cleaved by autoproteolysis to yield mature VP2 that forms the outer capsids of the virus. The VP2 is the major host-protective antigen of IBDV; it contains at least three independent epitopes responsible for the induction of neutralizing antibody (4). Segment A also encodes for the VP4 (protease), and the VP3 (inner capsid). Genome segment B encodes the virus polymerase VP1 (11).

Control of IBDV is currently attempted using live and killed vaccines for the dams and/or offspring (3). Immunization of chickens against IBDV with viral vectors expressing the VP2 has been previously reported and includes using avian herpesvirus (22), fowlpox virus (2), Newcastle disease virus (8) and fowl adenovirus (21) as expression systems. The advantages of the recombinant technology include safety (no reversion to pathogenicity of vaccine virus), that the vaccine can be tailor made to match field virus phenotype increasing its efficacy and that a vaccine based on VP2 alone should allow monitoring of the field situation by the discrimination between antibody induced by vaccine (anti-VP2 only) and that induced by infection (anti-VP2 and VP3) (10, 20).

The adeno-associated virus (AAV) is a replication defective virus member of the family *Parvoviridae* that has been successfully used for gene delivery in humans and other species (23).

The avian adeno-associated virus (AAAV) has been successfully used for transgenic expression of a reporter gene in chicken embryo tissues and cells (5, 6). Recent work demonstrated the feasibility of generating recombinant AAAV virions expressing the immunogenic VP2 peptides of the Edgar strain of IBDV (rAAAV-VP2) and that inoculation of specific pathogen free (SPF) birds with these recombinant virions generates serological evidence of VP2 expression *in vivo*(14). The aim of this work was to evaluate the protection against a virulent IBDV challenge in SPF chickens vaccinated with rAAAV-VP2 virions and to compare with commercial intermediate classic IBDV vaccination.

MATERIALS AND METHODS.

Viruses. The intermediate classic vaccine ViBursa CE (Lohmann Animal Health. Winslow, Maine. USA) was used as recommended by the manufactures. The Edgar strain used for the challenge was obtained from the Poultry Diagnostic and Research Center (Athens, GA. USA) stock and passed in three week-old SPF chickens bursas to obtain a titer of 10^5 chicken infectious dose 50 per ml (CID_{50}/ml).

The AAAV-VP2 virions expressing the immunogenic VP2 peptides of the Edgar strain of IBDV were generated as previously explained (14). Briefly, rAAAV virions were generated by simultaneous transfection of human embryo kidney cells (HEK 293) monolayers with three plasmid constructs: a plasmid designated p3.6 ITR-MCS-VP2, containing the Edgar strain VP2 gene under the influence of the late cytomegalovirus (CMV) promoter and flanked by the inverted terminal repeats (ITR) of the DA-1 strain of AAAV (Fig.1), a second plasmid expressing the Rep and Cap coding regions of AAAV designated pRC-CMV (6) and a commercial pHelper plasmid coding for the E2, E4 and VR-RNA genes derived from the human

adenovirus type 5 (Stratagene, La Jolla, CA. USA). The titer was determined by dot blot and reported as 1 x 10^{10} virus molecules/ml.

Experimental design. Ninety six, seven day-old SPF chickens were divided in six groups of 16 birds and placed in biosecurity level two units where adequate husbandry was provided. Two groups were vaccinated by the intramuscular route with 0.1 ml of the rAAAV-VP2 containing 10^9 virus molecules (groups 1 and 4). Another two groups (2 and 5) were vaccinated by eye drop with a commercial classic intermediate vaccine 10^5 CID_{50}/ml and the last two groups (3 and 6) remained as nonvaccinated controls. At 14, 21, 28, and 35 days of age, eight birds from each group were randomly selected and tested for the presence of anti-IBDV immunoglobulin G (IgG) using a commercial ELISA test (IDEXX Laboratories Inc., Westbrook, ME. USA), following manufacturer's protocols. On the same sampling days, two birds per group were sacrificed; body and bursa weights were individually recorded to obtain the bursa / body weight ratio (bursa weight / body weight x 100). Bursas were preserved in 10% buffered formalin for microscopic evaluation. At 28 days of age the birds in groups 1, 2 and 3 (rAAAV-VP2, commercial and nonvaccinated groups, respectively) were challenged by eye drop with 0.1 ml of virulent Edgar strain (10^4 CID_{50}/ml). The remaining birds (groups 4, 5, and 6) were not challenged and were used as treatment specific nonchallenged controls. All birds were observed twice a day for 7 days after challenge, in order to record IBDV clinical signs and mortality.

Histopathology. The extent of bursal histological damage was graded on a scale from 1 to 4 as previously described (19). Briefly, 1 = normal to 10% follicular atrophy; 2 = focal, mild scattered cell depletion up to 10-30% follicular atrophy; 3 = multifocal follicular atrophy 30-70%; 4 = diffuse atrophy of > 70% of the follicles or any evidence of acute necrosis.

Statistical analysis. All statistical analysis was performed using the Sigma Stat 3.0 software. Dunn's method and SNK test were performed at $P \leq 0.05$.

RESULTS AND DISCUSSION.

Previous reports indicate that recombinant avian adeno-associated viruses induce the expression of a reporter gene in tissues derived from the rAAAV inoculated embryos (5) and that inoculation of SPF birds with a rAAAV vector expressing the VP2 of IBDV (rAAAV-VP2) stimulate a systemic humoral immune response measured as IBDV specific ELISA titers (14). The ability of the immunity induced by rAAAV-VP2 to protect birds against infectious bursal disease remained to be clarified. In the present study we evaluated the level of protection conferred by rAAAV-VP2 vaccination in SPF chickens against a virulent challenge with the Edgar strain of IBDV. Morbidity measured as the presence of IBDV clinical signs, mortality, serological response, bursa/body weight ratio and the bursal lesion scores were used as criteria to assess protection; the results are summarized in Table 1.

Antibodies were detected by the IBDV specific ELISA test as early as day 14 in all vaccinated birds. Statistically significant differences were observed at 14, 21 and 28 days of age between the vaccinated groups and the nonvaccinated controls. The serological evidence of host recognition of the transgenic protein expression corroborates the suitability of the system for expression of poultry viruses immunogenic proteins (5, 14). When compared with the rAAAV-VP2 vaccinated birds the antibody levels induced by the commercial vaccine were higher at all sampling points. These disparities can be explained by host dependant differences in the immune mechanisms involved in the response to live IBDV replication (commercial vaccine) and the replication defective parvovirus used in the vector system (21).

Different levels of protection were observed within the challenged groups. In the nonvaccinated-challenged control group 100% of the birds showed clinical signs, ruffled feathers, sick birds and severe prostration were observed as early as day two after challenge, furthermore, 40% of the birds died within the observation period. The macroscopic lesions included edema and hemorrhagic bursas observed three days after challenge strain, similar lesions have been reported when SPF birds are challenged with the Edgar strain (19). These kinds of lesions are also commonly observed with the highly pathogenic strains distributed in Europe, Asia and Latin America (1, 7).

In the rAAAV-VP2 vaccinated-challenged group, two out of ten birds showed IBDV clinical signs and died, eight birds remained healthy during the duration of the experiment despite of the challenge. Due to the replication defective nature of the vector used, proper delivery to the host cells is required to ensure target protein expression, inadequate intramuscular inoculation or poor individual response to vaccination of this two SPF birds may account for lack of efficacy. Other possible explanations include a previously reported tendency of recombinant adeno-associated virus particles to agglutinate spontaneously, the aggregation of recombinant AAAV seems to be directly associated with variability in levels of empty capsids and DNA or protein impurities in the vector preparations, leading to reduced yield and less efficient gene transfer (23). In this case, due to the agglutination induced gene transfer decrease the threshold of protein expression required to mount an effective immune response may had not been reached in the two birds that were not protected, further experiments are required to clarify this point.

After challenge, the bursa/body weight ratios of the bird surviving in the rAAAV-VP2 group were significantly ($P<0.05$) higher and the bursal lesion scores lower than those of the commercial vaccinated and unvaccinated control groups, indicating adequate protection. None of

the birds inoculated with the commercial vaccine showed clinical IBDV or died after challenge, these results are in agreement with previous reports where Lukert strain derived vaccines efficacy against IBDV virulent challenge has been demonstrated (10, 15).

The histopathology of the bursas of Fabricius seven days after challenge is shown in Fig. 2. Nonchallenged groups are shown for comparison. The nonvaccinated-challenged group showed severe lymphoid depletion of the follicles, increased amount of stroma between follicles and severe follicular atrophy. The commercial vaccine group showed moderate lymphoid depletion in many follicles and some signs of lymphoid repopulation. The rAAAV-VP2 challenged group showed mild lymphoid depletion of the follicles, indicating no challenge derived tissue damage. Similar levels of protection have been previously reported for other recombinant vaccines expressing the immunogenic VP2 protein (2, 8, 20, 21).

The three groups that remained unchallenged (groups 4, 5, 6) showed no IBDV clinical signs during the entire experiment. When compared with their homologous challenged groups at day 35, the bursa/body weight ratios were higher and the bursal lesion scores lower, demonstrating the effect of the virus used in the challenge and validating the results obtained in the challenged groups. The commercial vaccine group showed moderate to severe lymphoid depletion of the follicles. No lesions were observed in the bursas from the rAAAV-VP2 vaccinated groups, in accord with what has been previously reported for other IBDV recombinant vaccines (2).

Live vaccines against IBDV have been proved to generate very good protection against the disease. Nevertheless, due to the worldwide emergence of very virulent IBDV the industry has resorted to the use of less attenuated vaccines that can compromise the bursal integrity of the young bird and may affect its ability to respond against other pathogens or standard vaccination programs (12, 17). The results indicate that albeit transgenic VP2 expression induces measurable

immune responses, the recombinant viruses do not target bursal B lymphocytes, representing the most important competitive advantage over live vaccines prepared using virus strains with increased pathogenicity (16, 17).

The bursal tissue integrity observed after rAAAV-VP2 vaccination correlates with the expectation of an adequate response to vaccination programs or field challenges after initial IBDV vaccination (18). Therefore, early and frequent vaccination with intermediate or intermediate plus vaccines can induce high levels of IBDV neutralizing antibodies, but can also deploy the bursal B cell population generating problems with immune responses against other pathogens and failure of vaccination programs (10, 18).

This pilot study only evaluated the protection after parenteral application and against classical IBDV viruses, nevertheless, initial investigations revealed a humoral immune response after *in ovo* application of rAAAV-VP2 particles (14). Also, the possibility of generating a broad spectrum vaccine by including both classical and variant VP2 proteins is sound. A tandem copy of the genes of interest with an internal ribosomal entry site will generate a bi-cistronic mRNA to express both classical and variant VP2 proteins. Although the 80% protection obtained in this trial is close to the 90% protection expected in an efficacy test for IBDV vaccines, the number of birds used in this proof of concept study was limited and further trials including a larger number of birds are required. Overall, this experiment demonstrated that the rAAAV based transgenic expression of the IBDV VP2 protein in SPF birds induces protective immunity against IBDV virulent challenge and that vaccination with the rAAAV-VP2 virions did not affect bursal lymphoid tissue, demonstrating its potential for use in young chickens.

Acknowledgements.

The authors will like to thank to Dr. Stanley Kleven, from the University of Georgia for reviewing this manuscript and for his valuable suggestions.

REFERENCES

1. Banda, A., and P. Villegas. Genetic characterization of very virulent infectious bursal disease viruses from Latin America. Avian Dis. 48:540-549. 2004.

2. Bayliss, C. D., R. W. Peters, J. K. Cook, R. L. Reece, K. Howes, M. M. Binns, and M. E. Boursnell. A recombinant fowlpox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus. Arch Virol. 120:193-205. 1991.

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

4. Coulibaly, F., C. Chevalier, I. Gutsche, J. Pous, J. Navaza, S. Bressanelli, B. Delmas, and F. A. Rey. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell. 120:761-772. 2005.

5. Estevez, C., and P. Villegas. Recombinant avian adeno-associated virus: transgene expression in vivo and enhancement of expression in vitro. Avian Dis. 50:216-221. 2006.

6. Estevez, C., and P. Villegas. Sequence analysis, viral rescue from infectious clones and generation of recombinant virions of the avian adeno-associated virus. Virus Res. 105:195-208. 2004.

7. Hassan, M. K. Very virulent infectious bursal disease virus in Egypt: epidemiology, isolation and immunogenicity of classic vaccine. Vet Res Commun. 28:347-356. 2004.

8. Huang, Z., S. Elankumaran, A. S. Yunus, and S. K. Samal. A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV. J Virol. 78:10054-10063. 2004.

9. Lombardo, E., A. Maraver, I. Espinosa, A. Fernandez-Arias, and J. F. Rodriguez. VP5, the nonstructural polypeptide of infectious bursal disease virus, accumulates within the host plasma membrane and induces cell lysis. Virology. 277:345-357. 2000.

10. Lukert, P. & Saif Y. Infectious bursal disease. In: Diseases of Poultry. H. J. Barnes. Y.M. Saif, J.R. Glisson, A.M. Fadly, L.R. Mc Dougald, D.E. Swayne., eds. Iowa State University Press, Ames, IA. pp 161-179. 2003.

11. Macreadie, I. G., and A. A. Azad. Expression and RNA dependent RNA polymerase activity of birnavirus VP1 protein in bacteria and yeast. Biochem Mol Biol Int. 30:1169-1178. 1993.

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

13. Muller, H., C. Scholtissek, and H. Becht. The genome of infectious bursal disease virus consists of two segments of double-stranded RNA. J. Virol. 31:584-589. 1979.

14. Perozo, F., P. Villegas., C. Estevez., I. Alvarado., L. Purvis. A recombinant avian adeno-associated virus as a vector for infectious bursal disease vaccination. Revista Cientifica FCV/LUZ. 17:423- 434. 2007.

15. Rautenschlein, S., and C. Haase. Differences in the immunopathogenesis of infectious bursal disease virus (IBDV) following in ovo and post-hatch vaccination of chickens. Vet Immunol Immunopathol. 106:139-150. 2005.

16. Rautenschlein, S., C. Kraemer, J. Vanmarcke, and E. Montiel. Protective efficacy of intermediate and intermediate plus infectious bursal disease virus (IBDV) vaccines against very virulent IBDV in commercial broilers. Avian Dis. 49:231-237. 2005.

17. Rautenschlein, S., H. Y. Yeh, and J. M. Sharma. Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic infectious bursal disease virus. Avian Dis. 47:66-78. 2003.

18. Rautenschlein, S., H. Y. Yeh, and J. M. Sharma. The role of T cells in protection by an inactivated infectious bursal disease virus vaccine. Vet Immunol Immunopathol. 89:159-167. 2002.

19. Rosales, A. G., P. Villegas, P. D. Lukert, O. J. Fletcher, M. A. Mohamed, and J. Brown. Pathogenicity of Recent Isolates of Infectious Bursal Disease Virus in Specific-Pathogen-Free Chickens: Protection Conferred by an Intermediate Vaccine Strain. *Avian Diseases*, 33: 729-734. 1989.

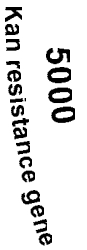
20. Shaw, I., and T. F. Davison. Protection from IBDV-induced bursal damage by a recombinant fowlpox vaccine, fpIBD1, is dependent on the titter of challenge virus and chicken genotype. Vaccine. 18:3230-3241. 2000.

21. Sheppard, M., W. Werner, E. Tsatas, R. McCoy, S. Prowse, and M. Johnson. Fowl adenovirus recombinant expressing VP2 of infectious bursal disease virus induces protective immunity against bursal disease. Arch Virol. 143:915-930. 1998.

22. Tsukamoto, K., S. Saito, S. Saeki, T. Sato, N. Tanimura, T. Isobe, M. Mase, T. Imada, N. Yuasa, and S. Yamaguchi. Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J Virol. 76:5637-5645. 2002.

23. Wright, J. F., G. Qu, C. Tang, and J. M. Sommer. Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. Curr Opin Drug Discov Devel. 6:174-178. 2003.

Fig. 5.1 Plasmid p3.6 ITR-MCS-VP2, containing the Edgar strain VP2 gene under the influence of the late cytomegalovirus (CMV) promoter and flanked by the inverted terminal repeats (ITR) of the DA-1 strain of AAAV.



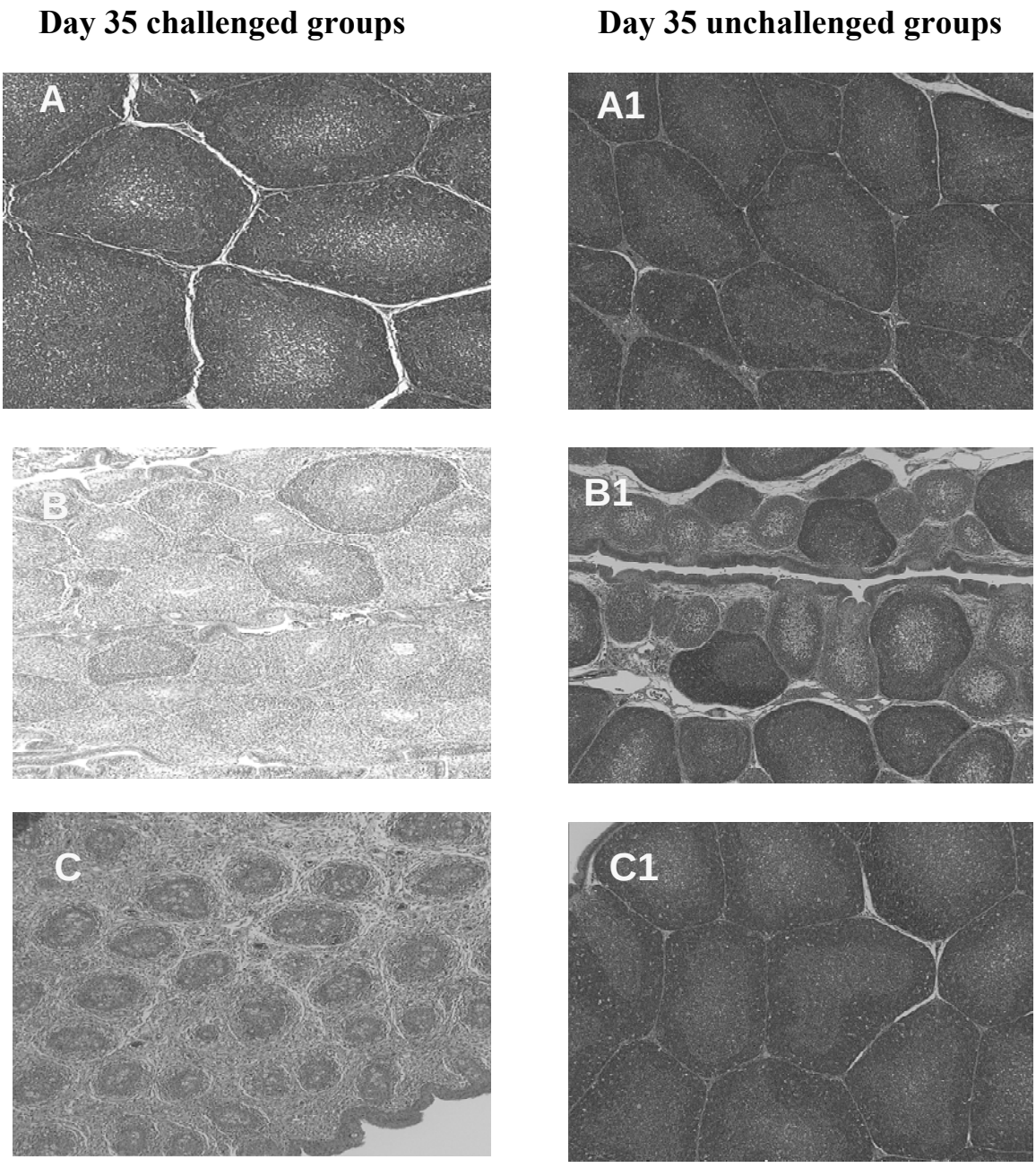


Fig 5.2. Histopathology of the Bursa of Fabricius at day 35. Challenged groups: rAAAVP2/challenged (A) = mild lymphoid depletion of the follicles. Commercial vaccine/challenged (B) = moderate lymphoid depletion of the follicles. Nonvaccinated/challendged (C) = severe lymphoid depletion of follicles, increased amounts of stroma between follicles and severe follicular atrophy. Nonchallenged groups: rAAAVP2 (A1) = no lymphoid depletion of the follicles. Commercial vaccine (B1) = lymphoid depletion in many follicles and lymphoid repopulation of some follicles. Nonvaccinated/nonchallenged (C1) = normal bursal histology. (H&E. 100X).

TABLE 5.1. Comparison of rAAAV-VP2 and commercial vaccine trial results.

	Day 14			Day 21			Day 28			Day 35			Clinical protection to challenge	
Treatment* **	**ELISA titers ***	**Bw/Bw Index****	**Bursa score**	**ELISA titers**	**Bw/Bw index**	**Bursa score**	**ELISA titers**	**Bw/Bw index**	**Bursa score**	**ELISA titers**	**Bw/Bw index**	**Bursa score**	**Morbidity %**	**Mortality %**
rAAAV-VP2 challenged at day 28	**775 [a]**	**0.53 [a]**	**-**	**920 [a]**	**0.60 [a]**	**1.0**	**1100 [b]**	**0.85 [a]**	**1.5**	**3250 [b]**	**0.54[b]**	**2.5**	**20**	**20**
Commercial challenged at day 28	**685 [a]**	**0.30 [b]**	**-**	**1049 [a]**	**0.28 [b]**	**3.0**	**2805 [a]**	**0.19 [b]**	**3.0**	**4581 [a]**	**0.19 [c]**	**3.0**	**0**	**0**
Control challenged at day 28	**0 [b]**	**0.55 [a]**	**-**	**0 [b]**	**0.73 [a]**	**1.0**	**0 [c]**	**0.63 [a]**	**1.5**	**2020 [a]**	**0.20[c]**	**4.0**	**100**	**40**
rAAAV-VP2 unchallenged	**599 [a]**	**0.54 [a]**	**-**	**796 [a]**	**0.55 [a]**	**1.0**	**980 [b]**	**0.63 [a]**	**2.0**	**1065 [a]**	**0.64 [a]**	**2.0**	**-**	**-**
Commercial unchallenged	**923 [a]**	**0.27 [b]**	**-**	**1043 [a]**	**0.33 [b]**	**3.0**	**2977 [a]**	**0.26 [b]**	**3.0**	**3100 [a]**	**0.24[c]**	**2.5**	**-**	**-**
Control unchallenged	**0 [b]**	**0.53 [a]**	**-**	**0 [b]**	**0.59 [a]**	**1.0**	**0 [c]**	**0.77 [a]**	**1.5**	**0 [a]**	**0.66 [a]**	**1.5**	**-**	**-**

* ELISA antibodies expressed as geometric mean (GMT) titers.

** Bursa / body weight ratios calculated using the following formula: Bursa weight / body weight x 100.

*** Means with the same letter within column are not significantly different by the SNK test ($P < 0.05$)

CHAPTER VI

THE VG/GA STRAIN OF NEWCASTLE DISEASE VIRUS: MUCOSAL IMMUNITY, PROTECTION AGAINST LETHAL CHALLENGE AND MOLECULAR ANALYSIS.

Perozo, F. P. Villegas, R. Dolz, C. L. Afonso & L. B. Purvis. **Accepted by Avian Pathology.** Reprinted here with permission of publisher.05/202/2008

Abstract.

The Villegas-Glisson / University of Georgia (VG/GA) strain of Newcastle disease virus (NDV) isolated from the intestine of healthy turkeys has been proposed to replicate in the respiratory and intestinal tract of chickens. In this study, the virus distribution, the mucosal and systemic immune response, the efficacy against lethal challenge and the full genome sequence of the VG/GA strain were compared with LaSota strain of NDV. The VG/GA strain was detected at different time points in the respiratory and intestinal tract of chickens with a preferential tropism for the latter. Both the VG/GA and LaSota strains induced NDV specific immunoglobulin A (IgA) at the upper respiratory tract. IgA levels were higher in the trachea for LaSota strain, while they were higher in the bile and intestine for the VG/GA strain. Positive correlation between virus distribution of the viruses and IgA production was observed. Despite the presence of the maternal antibodies in broilers, early vaccination with the VG/GA strain afforded 95 to 100% protection against lethal challenge, equivalent to the protection conferred by LaSota strain. Full genome sequence analysis classified the VG/GA strain within class II, genotype II viruses, which also include most of the respirotropic vaccine strains. Differences with the LaSota strain at the nucleotide and amino acid levels that may explain the differential phenotype of the VG/GA were observed; however, verification of the significance of those changes is required. Taken together, these results validate field observations on the efficacy of VG/GA vaccination and demonstrated the unique characteristics of the strain.

Introduction

Newcastle Disease virus (NDV) is one of the most important infectious agents in the poultry industry, affecting a wide variety of birds and causing important economical losses (Alexander, 2001). The virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, in the genus *Avulavirus* (Mayo, 2002). Vaccination of commercially reared birds is the best way to reduce losses resulting from NDV infection (Senne, *et al.*, 2004). For primary NDV vaccination, the vaccine of choice is one that elicits adequate immune response with minimal respiratory reactions (Villegas, 1998).

The Villegas-Glisson / University of Georgia (VG/GA) strain of NDV have been proposed to replicate both in the respiratory and intestinal tract, with preference for the intestine. Vaccine-induced respiratory reactions are decreased or avoided when using the VG/GA strain, probably by diminishing the level of replication in the respiratory epithelia of young chickens (Nunes, *et al.*, 2002). Initial investigations with the VG/GA strain stated that the virus produced no detectable respiratory reaction in chickens regardless of the vaccination route used, that it afforded respiratory tract protection against challenge with a respiratory-type NDV (LaSota strain) and it produced high hemagglutination inhibition (HI) titres showing no interference with immunity to infectious bronchitis (Glisson *et al.*, 1990).

Most of the commercially available lentogenic vaccines are able to induce antibodies against NDV; however, systemic humoral immune response measured as the presence of specific NDV antibodies in serum is not enough for protection (Erf, 2004; Reynolds & Maraqa, 2000). It has been established that the mucosal immunity represented by immunoglobulin A (IgA) production, play an important role in the development of protection in chickens vaccinated against Newcastle disease (Reynolds & Maraqa, 2000; Scott, 2004; Seal, *et al.*, 2000). Antibody

production in the mucosa is closely related to viral replication in the target cells, hence the pathogenesis and tissue tropism of the viruses used for vaccination is to be considered in order to assess the efficacy of a given live vaccine against a direct challenge (Jayawardane & Spradbrow, 1995). The VG/GA strain's intestinal tropism and the consequent induction of local immunity may be important for protection against velogenic-viscerotropic strains of NDV that have been reported to induce massive destruction of intestinal lymphoid areas and extensive ulceration of overlying intestinal epithelium (Brown, *et al.*, 1999).

The VG/GA strain, when applied to immune competent specific pathogen free (SPF) chickens, induces protection against lethal NDV challenge (Beard, *et al.*, 1993). Also, anecdotal data obtained from field experiences indicate that the VG/GA strain is useful in the control of velogenic-viscerotropic NDV strains in poultry. Full protection against lethal challenge has been reported when the VG/GA strain is included in vaccination programs under experimental and field conditions (Perozo, 2004; Silva, 2004).

The reason why the VG/GA and some asymptomatic enteric strains preferentially target intestinal epithelia cells is unknown. Changes in the structural proteins like the polymerase protein (L), the fusion (F) protein or the hemagglutinin-neuraminidase (HN) protein have been recently reported as major determinants of tropism and virulence (de Leeuw, *et al.*, 2005; Huang, *et al.*, 2004) and could explain the differential tissue tropism observed among lentogenic strains. The HN and F proteins may determine a unique attachment and fusion phenotype that allows the enterotropic viruses to preferentially enter the intestinal cells and the differences in the polymerase (L) protein may allow higher levels of replication for the enterotropic strains or impair the respirotropic strains replication in the intestine.

In this study, virus distribution, local and systemic humoral immune response to in SPF birds and the protection against lethal challenge conferred by vaccination with the VG/GA strain in commercial broiler chickens were evaluated and compared with LaSota strain of NDV. In addition, complete nucleotide sequencing and full genome analysis of the VG/GA strain were performed to assess the genomic bass of the strain phenotype.

Materials and Methods.

Viruses. The NDV LaSota strain used in the study was obtained from a commercial vaccine (Merial Select, Inc. Gainesville, Georgia. USA). The VG/GA/turkey/1987 strain used was the original isolate (embryo passage 3). Both strains were propagated by inoculation into embryonating chicken eggs and titres of 10^8 embryo infective dose 50/ml (EID_{50}/ml) were obtained for both viruses. The AF stock virus was kept at -80 C until used.

Experimental design. To test mucosal and systemic humoral immune response to vaccination, ninety 1 day-old specific pathogen free (SPF) chickens (Sunrise Farms, Catskill. NY.) were placed in groups of 30 in biosecurity level 2 isolation units where appropriated husbandry was provided. At ten days of age, each group was inoculated by the oral/ocular route with 0.1 ml of distilled water, or with a total dose of 10^7 EID_{50} in 0.1 ml of the VG/GA or LaSota strains, respectively.

Systemic humoral immune response. At 1, 4, 7, 11, 15 and 21 days post inoculation, eight birds in each group were bled and the serum samples used for enzyme linked immunosorbent assay (ELISA) assessment using a commercial Newcastle disease antibody test (FlockCheck®, IDEXX , Maine, USA).

NDV-specific IgA detection. In each of the above mentioned sampling days, four birds per group were selected to obtain tracheal and intestinal washes and bile. Briefly, chickens were

euthanized using a CO_2 chamber, the tracheas were clamped below the syrinx and 0.4 ml of phosphate buffer saline flushed in and out 10 times. Similar procedure was applied to a 10 cm segment of the duodenum that was separated and clamped at one end. Bile was obtained by direct puncture of the gall bladder. Samples were placed in sterile containers and processed fresh. NDV-specific IgA levels in tracheal and intestinal lavages and bile were assayed by duplicate using an indirect ELISA as described elsewhere (Raj & Jones, 1996), except that the coating antigen used was LaSota strain of NDV and that the chicken IgA binding to the coating antigen was detected with a commercial goat anti-chicken IgA reagent (alpha chain specific), conjugated with horse radish peroxidase (Bethyl Laboratories, Inc. Montgomery, USA.). The optical density at 650 (OD650) was measured using a Precision microplate reader (Molecular Devices. Inc. New York, USA). Corrected optical density values were calculated by subtracting the optical density values of non-antigen coated-wells from those of test wells.

Total IgA detection. To estimate the total (unspecific) IgA production in the respiratory and intestinal mucosa, a commercial ELISA chicken IgA quantitation kit (Bethyl Laboratories, Inc. Montgomery, Texas. USA.) was used on tracheal washings and bile samples following the manufacture's recommendations. Checkerboard titrations established an optimal conjugate dilution of 1/10,000.

Viral tissue distribution. Tissue samples were collected from trachea, lung, duodenum and cecal tonsils at 1, 4, 7, 11, 15 and 21 days post inoculation and kept frozen at -80 C until processing. After enzymatic digestion (proteinase K) of the individual samples, the tissue tropism of the strains was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) using the high pure RNA tissue kit (Roche Diagnostics Co., Indianapolis, IN) for RNA extraction and the one step RT-PCR Kit (Titan-Roche Diagnostics Co) for amplification. NDV

specific primers and protocols were used as described elsewhere (Seal *et al.,* 1995). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

VG/GA strain vaccine-challenge trial. A total of 288 one day-old broiler chickens (Merial Select, Gainsville, GA. USA) with average maternal antibody ELISA titres of 1840, were randomly divided into nine groups of 32 birds each and vaccinated by eye drop at days 1 and/or 14 with a total dose of 10^7 EID_{50} in 0.1 ml of the VG/GA and/or LaSota strains, one group remained as unvaccinated control. The treatment combinations are shown in Table 1. On days 14, 21, and 28 the NDV specific ELISA test was performed on serum samples from eight birds per group. The IgA levels in trachea washings and bile were measured each sampling day in four birds per group as described above. On day 28 of the experiment the birds were challenged by the intramuscular route with a dose of 10^4 EID_{50} per bird of the velogenic Texas GB strain of NDV.

VG/GA strain genome amplification. To assess the genomic base of the VG/GA strain tissue tropism, a primer sequence independent amplification method was used on 200 μl of VG/GA stock allantoic fluid as described elsewhere (Djikeng, 2006).

Sequence data, nucleotide sequencing and alignment analysis. All nucleotide sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated ABI sequencer (ABI 3700 automated sequencer; Applied Biosystems Inc., Foster City, CA). Nucleotide sequence assembly and editing were conducted with the CodonCode sequence analysis software package. Comparison sequences were retrieved from GenBank public databases and used to generate alignments. Accession numbers for the 28 full genome NDV sequences used for comparison and alignment are as follows: AF375823, NC002617, AF309418,

Y18898, AY845400, AF077761, DQ60053, AY225110, AY562991, DQ097394, AY935499,AY935500, AY935498, AY935489, AY741404, AY562990, AY562986, AY562987, AJ880227, AY562989, AY562988, AY865652, AY562285, DQ659677, DQ485231, DQ485230, DQ485229, AF431744.

Alignments of complete genomes were performed using BioEdit v. 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh) with the ClustalW program followed by manual editing. Phylogenetic tree construction was done with Phyml V2.4.4 (for bootstrap analysis) under the GTR model of nucleotide substitution with estimated proportions of invariable sites, ML base frequencies estimates, four substitution rate categories, and an affixed gamma distribution parameter. Coding regions were identified by translating the corresponding open reading frame with Bioedit and proteins alignments were performed with the ClustalW program.

Statistical analysis: All statistical analysis was performed using the Sigma Stat 3.0 software. Dunn's method and SNK test were performed at $P \leq 0.05$.

Results.

Serum IgG levels of VG/GA and LaSota vaccinated SPF birds. The profile of the ELISA results obtained at the different sampling time points from the SPF birds vaccinated with VG/GA or LaSota strais of NDV corresponds with a primary progresive immune response regardless of the vaccine virus used (Figure 1). Both viruses induced IgG antibody levels that were statistically different ($P<0.05$) from the unvaccinated control groups at days 11, 15 and 21 post vaccination. No statistical differences within the strains tested were observed.

NDV-specific IgA levels in biological samples of VG/GA and LaSota vaccinated SPF birds. The IgA levels detected in the tracheal and intestinal washings and bile are shown in figures 2A, 2B and 2C respectively. The tracheal washings, representing the respiratory component of the mucosal immune response, showed that both viruses were able to induce measurable levels of NDV-specific IgA. LaSota strain of NDV induced overall higher levels of IgA when compared with the VG/GA strain, however both groups differed ($P< 0.05$) from the unvaccinated controls. For the bile samples, higher IgA levels ($P<0.05$) were observed in the birds vaccinated with the VG/GA strain of NDV. Similar results were observed for the intestinal washings.

Total IgA in biological samples of VG/GA and LaSota vaccinated SPF birds. No significant differences ($P<0.05$) were observed in the levels of total (unspecific) IgA between the control and the vaccinated groups in all the samples tested (data not shown).

RT-PCR detection of NDV in respiratory and intestinal tissues of SPF birds.

To confirm the ability of VG/GA strain to replicate in the respiratory and intestinal tract of chickens, the virus distribution of the VG/GA was assessed by RT-PCR in SPF chickens vaccinated at 10 days of age and compared with the tissue tropism of LaSota strain. Results are shown in Table 2. The VG/GA strain was detected both in the respiratory and in the intestinal tract, with a preferential tropism for the later. The presence of the VG/GA in the trachea and lungs was transient and detectable only at 4 and 7 days post inoculation. As early as 24 hours after infection, the VG/GA nucleic acids were detected in the duodenum and remained detectable until day 11, in the cecal tonsils the RNA was amplified from 4 to 15 days post inoculation. The RT-PCR confirmed the respirotropic nature of LaSota strain by amplifying the virus from day one up to day 11 in the respiratory tissues.

Vaccine challenge trial. The different vaccination schedules assessed resulted in protection against lethal challenge, all the unvaccinated-challenged controls died within the observation period. The results for the experimental vaccine-challenge trial and the immunoglobulin measurements are summarized in Table 1. It was demonstrated that despite the presence of the maternal antibodies, early vaccination with the VG/GA in broilers generates systemic and mucosal NDV-specific antibodies accompanied by protection levels between 95 and 100% against a lethal velogenic challenge. The VG/GA efficacy was equivalent to the protection offered by LaSota strain. Combinations of the two strains were also highly efficacious. Systemic immune response varied among groups. Regardless of the vaccine strain administered first, the treatments with two doses induced higher antibody titers than those were only one vaccine was applied. All the vaccinated groups significantly differed ($P<0.05$) from the unvaccinated controls at days 21 and 28 post inoculation. The results for the IgA levels in respiratory and intestinal tract in the broiler chickens were equivalent to those observed in the SPF birds: higher IgA levels in the trachea washings for the LaSota strain and higher IgA levels in the intestinal tract of the VG/GA vaccinated birds.

Complete nucleotide sequence of VG/GA strain. Phylogenetic comparison of the VG/GA genome with 28 NDV full genome sequences available in the GenBank is shown in Figure 3. The analysis, based on the full genome sequences indicates that the VG/GA strain can be grouped within the class II, genotype II which corresponds to most of the respirotropic vaccine strains used in the poultry industry and differed from the other lentogenic strains with enteric tropism (QV4, Ulster, PHY-LMV42) that belong to the class II in the genotype I.

Differences were observed at both the nucleotide and amino acid levels when the VG/GA and the LaSota strains genes and proteins were compared. A total of 66 residue substitutions

were distributed in all the six structural proteins analyzed. Four amino acid changes where observed between the VG/GA and LaSota F proteins, the first of them, V-106-M, was located near the heptad repeat 4 (HR4) domain in the F_2 polypeptide. The second and third were contiguous changes (I-135-M and T-136-A) located at the end of the 20 extensively hydrophobic N-terminal residues of F_1.

The differences among the strains were located in the globular head of the HN and included substitutions of hydrophobic residues with polar residues; for instance a small non polar Glycine at position 169 in LaSota is substituted by an large polar Arginine residue in the VG/GA strain HN protein and a large non polar Valine is substituted by a small polar Glutamate. The length of the HN protein of the VG/GA strain was 577 amino acids.

The nucleotide sequence comparison of the VG/GA strain L gene revealed a single nucleotide insertion at position 3870. This mutation is compensated by a nucleotide deletion downstream at position 3958 which results in a 30-amino acid substitution in the domain V of the L protein. The mutation is present in the VG/GA and absent in the LaSota strain. At least five amino acid substitutions were observed in each of the remaining proteins: matrix, nucleocapsid and phosphoprotein. All differences are summarized in Table 3.

Discussion.

The RT-PCR results demonstrated that the VG/GA strain can be detected both in the respiratory and intestinal tract of chickens, while LaSota strain was detected in the respiratory tract. Although we did not perform virus isolation from the different tissue samples a very high positive correlation between virus isolation and RT-PCR detection that validates the use RT-PCR for NDV tissue tropism studies has been reported (Ganapathy*, et al.*, 2005; Gohm*, et al.*, 2000; Wakamatsu*, et al.*, 2007). An association between the site of replication and the levels of IgA production was

observed and can be explained by the virus replication and host antigen recognition mechanisms proposed for NDV (Alexander, 2001; de Leeuw *et al.,* 2005; Peeters, *et al.,* 1999). Active replication in the mucosa induces virus protein production and local antigen presentation through MHC class I and II molecules stimulating a T dependent B cell response at the site of infection in the form of IgA producing plasma cells in the intestine (Al-Garib*, et al.*, 2003).

The importance of local antibodies in the defence mechanism against viral infection has been emphasized in recent years (Scott, 2004). It appears that local immunity acts as a barrier at surfaces where primary viral infections occurs, thereby interfering with further spread of the virus (Jayawardane & Spradbrow, 1995; Russell. 1993). Birds have a well-developed mucosal immune system; its characteristics include local production and secretion of IgA antibodies and traffic of IgA producing plasma cells (Al-Garib*, et al.*, 2003; Jayawardane & Spradbrow, 1995; Zigterman*, et al.*, 1993). The IgA class predominates and is detectable in tears, saliva, tracheal and intestinal washes and bile. In this trial, the mucosal immune response measured as the levels of IgA induced by VG/GA strain vaccination was assessed. The replication pattern of VG/GA strain induced a stronger localized mucosal immune response in the intestinal tract shown by an increased production of NDV-specific IgA. This feature may represent a competitive advantage in the event of a velogenic viscerotropic challenge where the virus have been reported to induce massive destruction of intestinal lymphoid areas and extensive ulceration of overlying intestinal epithelium associated with active viral replication (Brown*, et al.*, 1999).

No significant differences were observed in the levels of total (unspecific) IgA between the nonvaccinated control and the vaccinated groups, suggesting that there is no measurable effect of NDV vaccination in the overall IgA load of the mucosal tissue in chickens. This observation could be explained as the consequence of commensal and/or pathogenic colonization

of the respiratory and intestinal epithelial surfaces which represent the putative site of initial antigen encounter (Brandtzaeg, 2003). Furthermore, epithelial cells have been proved to provide co-stimulatory signals promoting terminal differentiation of B-cells oriented towards IgA production, generating relatively high and constant levels of the immunoglobulin (Brandtzaeg, 2003; Scott 2004).

Systemic humoral immunity represented by neutralizing IgG antibodies against NDV hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins is a relevant component of the bird's protection against infection. Antibody based virus neutralization, complement activation and immune complex formation pathways are important for the control of NDV and correlate positively with protection (Seal*, et al.*, 2000). The trend observed in the serum IgG levels for the VG/GA and LaSota viruses corresponds with a primary immune response with progresive time dependant increase of the antibody titres.

Despite the presence of the maternal antibodies in broilers, early vaccination with the VG/GA strain afforded 95 to 100% protection against lethal challenge, equivalent to the protection offered by LaSota strain. These results validate anecdotic data obtained from field observations and confirm results from vaccine-challenge trials performed in SPF chickens (Beard*, et al.*, 1993) and in quails (Silva, 2004). The efficacy of the different treatment combinations using both VG/GA and LaSota strains, demonstrated the feasibility of using a multiple strain vaccine protocol with VG/GA strain for initial vaccination when high challenge is present and field revaccination is scheduled.

Based on how fast the VG/GA is cleared from the respiratory tract after priming the mucosal immune response, the mucosal IgA production both in the respiratory and the intestinal tract and the levels of protection afforded by single or multiple doses of the vaccine, initial

vaccination with the VG/GA strain may be advantageous for the integrity of the respiratory mucosa of young chickens when multiple vaccination and field exposure is expected. Previous reports on morphometric analysis and comparison of tracheal thickness after vaccination with different vaccine strains indicated that LaSota and Ulster strains had equivalent virulence and both caused higher swelling of tracheal mucosa than VG/GA strain (Nunes et al., 2002).

Antigenic (Alexander, *et al.*, 1998) and genetic diversity (Aldous, *et al.*, 2003), are recognized within the NDV isolates which are all members of the APMV-1 serotype. Based on nucleotide sequence there have been at least six distinct lineages identified for NDV (Aldous, *et al.,* 2003). A more traditional classification using full length sequence has been reviewed and comprises two major divisions represented by class I and class II, with class II being further divided into nine genotypes (Czegledi, *et al.*, 2006). For instance, the United States isolates of NDV identified in the decade of 1940's and most of the respirotropic vaccines used today to control Newcastle disease are class II, genotype II. After full genome sequence analysis, the VG/GA strain was grouped within the class II, genotype II. These results are in agreement with previous reports based on partial sequences (Aldous, *et al.*, 2003; Seal, *et al.*, 1995). The VG/GA phylogeny differed from the other vaccine strains able to replicate in the intestine; these strains belong to the class II but in the genotype I. The Ulster, QV4 derivates, and PHY-LMV42 strains are more closely related than the VG/GA to the Australian isolates for which drift mutations at the cleavage site responsible for increases in virulence has been reported. Over time some viruses in this group have acquired more basic amino acids and the leucine (L) change to phenylalanine (F) which made the final virus highly virulent (Gould, *et al.*, 2001).

The nucleotide and amino acid composition of the VG/GA strain were compared with the LaSota strain, differences were observed at both levels. The F glycoprotein of NDV is a type I

integral membrane protein that has been shown to be involved in virus penetration and cell fusion (Morrison, 2003). The amino acid changes between the F proteins of the VG/GA and LaSota strains included contiguous substitutions in the fusion peptide, which is conserved among paramyxovirus (up to 90% identity) and is directly involved in fusion promotion (Horvath & Lamb, 1992). The mutations observed in the fusion peptide of the VG/GA strain F protein are unique and may be associated with its phenotype.

The nucleotide and amino acid changes between the VG/GA and LaSota HN protein were located in the globular head of the HN that has been proposed to be a dynamic molecule that switches from one conformational state to another, resulting in a change of an active site which is responsible for both receptor binding and neuraminidase activity (de Leeuw, *et al.*, 2005). Amino acid substitutions at specific locations can have a profound effect on the folding and function of the proteins and could be responsible for the differential tropism of these two strains. Romer-Oberdofer and colleges (Romer-Oberdorfer, *et al.*, 2003), indicated in 2003 that the length of the HN protein may play a role in the ability of the virus to spread and propagate in various organs after inoculation. The 577 amino acids of the VG/GA HN protein is also the length of the LaSota HN protein and has been reported for both virulent and avirulent strains, which may disregard the role of the protein size in the tissue tropism of the VG/GA strain.

The mutation in the domain V of the L protein present in the VG/GA has been previously documented, the authors reported the existence of two forms of the L protein of NDV after sequence analysis of NDV isolates from different backgrounds (Kusumaningtyas, *et al.*, 2004). The association of this genotype with virus replication and tissue tropism is yet to be determined for NDV; however, domain V has been proposed to play an important role in transcription and thermosensitivity of isolates of vesicular stomatitis virus and Sendai virus (Banerjee, 1987;

Cortese, *et al.*, 2000). An interesting observation is that the clone 30 strain which is a more attenuated from of LaSota strain of NDV (lower post-vaccine reactions) has the same amino acid profile as the VG/GA strain in this segment of the L protein (Romer-Oberdorfer, *et al.*, 1999). The changes observed in proteins associated with tissue tropism may explain the differential phenotype of the VG/GA strain; however, further studies including the generation of a reverse genetic system and nucleotide substitution studies are required to verify the significance of these changes.

Acknowledgements.

The authors will like to thank to Drs. Rafael Fernandez and Julio Cruz from Merial, US. and Dr. Carlos Estevez from the Southeast Poultry Research Laboratory, USDA for their support and valuable suggestions.

References

Al-Garib, S.O., Gruys, E., Gielkens, A.L. & Koch, G. (2003). Detection of antibody-forming cells directed against Newcastle disease virus and their immunoglobulin class by double immunoenzyme histochemistry. *Avian Diseases*. *47*, 453-7.

Aldous, E.W., Mynn, J.K., Banks, J. & Alexander, D.J. (2003). A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. *Avian Pathology*. *32*, 239-56.

Alexander, D.J. (2001). Gordon Memorial Lecture. Newcastle disease. *British Poultry Science*. *42*, 5-22.

Alexander, D.J., Morris, H.T., Pollitt, W.J., Sharpe, C.E., Eckford, R.L., Sainsbury, R.M., Mansley, L.M., Gough, R.E. & Parsons, G. (1998). Newcastle disease outbreaks in domestic fowl and turkeys in Great Britain during 1997. *Veterinary Records*. *143*, 209-12.

Banerjee, A.K. (1987). The transcription complex of vesicular stomatitis virus. *Cell*. *48*, 363-4.

Beard, C.W., Villegas, P. & Glisson, J.R. (1993). Comparative efficacy of the B-1 and VG/GA vaccine strains against velogenic viscerotropic Newcastle disease virus in chickens. *Avian Diseases*. *37*, 222-5.

Brandtzaeg, P. (2003). Mucosal immunity. *Developments in Biological Standardization (Basel)*. *115*, 111-7.

Brown, C., King, D.J. & Seal, B.S. (1999). Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. *Veterinary Pathology*. *36*, 125-32.

Cortese, C.K., Feller, J.A. & Moyer, S.A. (2000). Mutations in domain V of the Sendai virus L polymerase protein uncouple transcription and replication and differentially affect replication in vitro and in vivo. *Virology*. *277*, 387-96.

Czegledi, A., Ujvari, D., Somogyi, E., Wehmann, E., Werner, O. & Lomniczi, B. (2006). Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus Research*. *120*, 36-48.

de Leeuw, O.S., Koch, G., Hartog, L., Ravenshorst, N. & Peeters, B.P. (2005). Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. *Journal of General Virology*. *86*, 1759-69.

Djikeng, A., Halpin, R., Sengamalay, N., Ghedin, E. & Spiro, D. (2006). Application of the sequence-independent single primer amplification (SISPA) method for viral genome sequencing. *Proceedings of the Genomes, Medicine, and the Environment 2006 meeting* (p. 63). Hilton Head. USA.

Erf, G.F. (2004). Cell-mediated immunity in poultry. *Poultry Science*. *83*, 580-90.

Ewert, D.L., Barger, B.O. & Eidson, C.S. (1979). Local antibody response in chickens: analysis of antibody synthesis to Newcastle disease virus by solid-phase radioimmunoassay and immunofluorescence with class-specific antibody for chicken immunoglobulins. *Infection and immunology*. *24*, 269-75.

Ganapathy, K., Cargill, P., Montiel, E. & Jones, R.C. (2005). Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. *Avian Pathology*. *34*, 297-302.

Glisson, J., Villegas, P., Dufour, L., Christensen, L. & Page, D. (1990). Characterization of VG/GA Newcastle Disease virus as a vaccine candidate. *Proceedings of the 25th National Meeting on Poultry Health and Condemnations.*, (p.59), Ocean City, USA.

Gohm, D.S., Thur, B. & Hofmann, M.A. (2000). Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. *Avian Pathology*. *29*, 143-152.

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A. & Westbury, H.A. (2001). Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. *Virus Research*. *77*, 51-60.

Horvath, C.M. & Lamb, R.A. (1992). Studies on the Fusion Peptide of a Paramyxovirus Fusion Glycoprotein - Roles of Conserved Residues in Cell-Fusion. *Journal of Virology*. *66*, 2443-2455.

Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D. & Samal, S.K. (2004). The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. *Journal of Virology*. *78*, 4176-84.

Jayawardane, G.W. & Spradbrow, P.B. (1995). Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. *Veterinary Microbiology*. *46*, 69-77.

Kim, J.A., Ryu, S.Y. & Seo, S.H. (2005). Cells in the respiratory and intestinal tracts of chickens have different proportions of both human and avian influenza virus receptors. *Journal of Microbiology*. *43*, 366-9.

Kusumaningtyas, E., Tan, W.S., Zamrod, Z., Eshaghi, M. & Yusoff, K. (2004). Existence of two forms of L protein of Newcastle disease virus isolates due to a compensatory mutation in Domain V. *Archives of Virology*. *149*, 1859-65.

Mayo, M.A. (2002). Virus taxonomy - Houston 2002. *Archives of Virology*. *147*, 1071-6.

Morrison, T.G. (2003). Structure and function of a paramyxovirus fusion protein. *Biochimica et Biophysica Acta*. *1614*, 73-84.

Nunes, J., Vasconcelos, A.C., Jorge, M.A., Guimaraes, E.B., Paixao, T.A., Martins, N.R.S. & Resende, J.S. (2002). Comparative morphometric analysis of vaccinal virulence of some lentogenic strains of Newcastle disease virus in tracheas of SPF chickens. *Arquivo Brasileiro de Medicina Veterinaria y Zootecnia*. *54*, 335-339.

Peeters, B.P., de Leeuw, O.S., Koch, G.& Gielkens, A.L. (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *Journal of Virology*. *73*, 5001-9.

Perozo, F., Nava, J. & Rivera, S. (2004). Evaluation of two vaccination programs against Newcastle disease in Ross line broiler chickens reared under field conditions in Zulia state, Venezuela. 2. Immune response and protection against an experimental challenge. *Revista cientifica FCV/LUZ*. *14*, 387-394.

Raj, G.D. & Jones, R.C. (1996). Local antibody production in the oviduct and gut of hens infected with a variant strain of infectious bronchitis virus. *Veterinary Immunology and Immunopathology*. *53*, 147-61.

Reynolds, D.L. & Maraqa, A.D. (2000). Protective immunity against Newcastle disease: the role of cell-mediated immunity. *Avian Dis*eases *44*, 145-54.

Romer-Oberdorfer, A., Mundt, E., Mebatsion, T., Buchholz, U.J. & Mettenleiter, T.C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *Journal of General Virology*. *80,* 2987-95.

Romer-Oberdorfer, A., Werner, O., Veits, J., Mebatsion, T. & Mettenleiter, T.C. (2003). Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. *Journal of General Virology*. *84*, 3121-9.

Russell, P. H. Newcastle disease virus: virus replication in the harderian gland stimulates lacrimal IgA; the yolk sac provides early lacrimal IgG. Vet Immunol Immunopathol 37:151-163. 1993.

Scott, T. R. (2004). Our current understanding of humoral immunity of poultry. *Poultry Science*. *83*, 574-9.

Seal, B.S., King, D.J. & Bennett, J.D. (1995). Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *Journal of Clinical Microbiology. 33*, 2624-30.

Seal, B.S., King, D.J. & Sellers, H.S. (2000). The avian response to Newcastle disease virus. *Developmental and Comparative Immunology*. *24*, 257-68.

Senne, D.A., King, D.J. & Kapczynski, D.R. (2004). Control of Newcastle disease by vaccination. *Developments in Biological Standardization (Basel)*. *119*, 165-70.

Silva, F., Santin, E., Pulillo, A., Doretto, L., Barbosa, V., Queiroz, N. & Schocken-Iturrino, R. (2004). Evaluation of different programs of Newcastle Disease Vaccination in Japanese Quail (Coturnix cotournix japonica). *International Journal of Poultry Science. 3*, 354-356.

Villegas, P. (1998). Viral diseases of the respiratory system. *Poultry Science*. *77*, 1143-5.

Wakamatsu, N., King, D.J., Seal, B.S. & Brown, C.C. (2007). Detection of Newcastle disease virus RNA by reverse transcription-polymerase chain reaction using formalin-fixed, paraffin-

embedded tissue and comparison with immunohistochemistry and in situ hybridization. *Journal of Veterinary Diagnostic Investigations*. *19*, 396-400.

Zigterman, G.J., van de Ven, W., van Geffen, C., Loeffen, A.H., Panhuijzen, J.H., Rijke, E.O. & Vermeulen, A.N. (1993). Detection of mucosal immune responses in chickens after immunization or infection. *Veterinary Immunology and Immunopathology*. *36*, 281-91.

Table 6.1. NDV V*accine-challenge trial local and systemic humoral immunity, and protection against lethal challenge.*

GROUP	AGE (DAYS 14			21			28[C]			42
	ELISA[a] Titers	Trachea[b] NDV IgA	Bile IgA	ELISA Titers	Trachea NDV IgA	Bile NDV IgA	ELISA Titers	Trachea NDV IgA	Bile NDV IgA	Challenge results: # of dead (% protection)
VG/GA day 1	134[A]	0.45[B]	0.93[A]	403[B]	1.02[B]	1.16[A]	1172[B]	1.21[B]	1.96[A]	(1 /19) 95%
VG/GA days 1+14	62[A]	0.41[B]	1.01[A]	261[C]	1.04[B]	1.57[A]	1865[A]	1 .18[B]	2.41[A]	0 /18 (100%)
VG/GA day 14	25[A]	0.05[C]	0.02[C]	47[C]	0.19[C]	0.31[B]	952[B]	0.90[B]	1.40[B]	0 /20 (100%)
LaSota day 1	271[A]	0.80[A]	0.42[B]	1049[A]	1.33[A]	0.42[B]	1578[A]	1.76[A]	1.04[B]	0 /19 (100%)
LaSota days 1+14	209[A]	0.72[A]	0.39[B]	1231[A]	1.23[A]	0.57[B]	1913[A]	1.98[A]	1.27[B]	0 /19 (100%)
LaSota day 14	5[A]	0.02[C]	0.06[C]	483[C]	0.65[B]	0.21[C]	874[B]	1.82[A]	0.42[C]	0 /19 (100%)
VG/GA day 1 + LaSota 14	92[A]	0.40[B]	1.01[A]	437[B]	0.72[B]	1.81[A]	1514[A]	1.44[B]	2.13[A]	0 /20 (100%)
LaSota day 1 + VG/GA 14	216[A]	0.74[A]	0.48[B]	547[B]	1.44[A]	1.74[A]	1485[A]	1.67[A]	2.29[A]	1 /19 (95%)
Control challenged	24[A]	0.05[C]	0[C]	22[C]	0.07[C]	0[C]	42[C]	0.05[C]	0.1[C]	9 /9 (0%)

[a] Eight birds per group were tested and results are expressed as the GMT of the ELISA titres. [b]Four birds per group were sampled for local immunity; results are expressed as the average of the corrected optical densities (COD). [c]Birds were challenged with a lethal dose of Texas GB strain at 28 days of age and observed for 14 days. Same capital letters within columns indicate no significant differences ($P<0.05$).

Table 6.2. *Reverse transcriptase-polymerase chain reaction detection of virus tissue distribution.*

RT-PCR	Days post inoculation / Organs																							
	1				4				7				11				15				21			
Treatments	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct
VG/GA	-	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	-	-	-
LaSota	+	-	-	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Control	**ALL TISSUES NEGATIVE**																							

Samples from four birds where obtained and pooled each day. T, trachea; L, lung; D, duodenum; Ct, cecal tonsil; +, RT-PCR positive signal (250 bp amplicon).; -, no amplification.

Table 6.3. *Amino acid changes among VG/GA and LaSota strains of NDV.*

Protein	Amino acid LaSota	Residue position	Amino acid VG/GA
HN			
	G	169	R
	Y	203	H
	S	324	T
	V	495	E
	T	522	I
F			
	V	106	M
	I	135	M
	T	136	A
	V	255	I
M			
	G	29	D
	L	48	S
	I	104	V
	I	114	M
	V	196	I
N	I	90	L
	M	389	R
	K	390	R
	D	401	E
	D	402	V
	I	407	D
P			
	E	8	D
	F	109	L
	P	164	L
	T	317	I
	K	352	R
L			
	P	18	S
	Q	97	E
	M	187	I
	K	191	R
	T	253	M
	T	305	I
	S	897	P
L (V domain)	CHLTFTYPMILKGCSL KKESKRGMWFTNRV	1287-1316	VSPYIHISNDSQRLF TEEGVKEGNVVYQQI
	V	1366	A
	S	1385	L
	E	1620	G
	I	2103	K

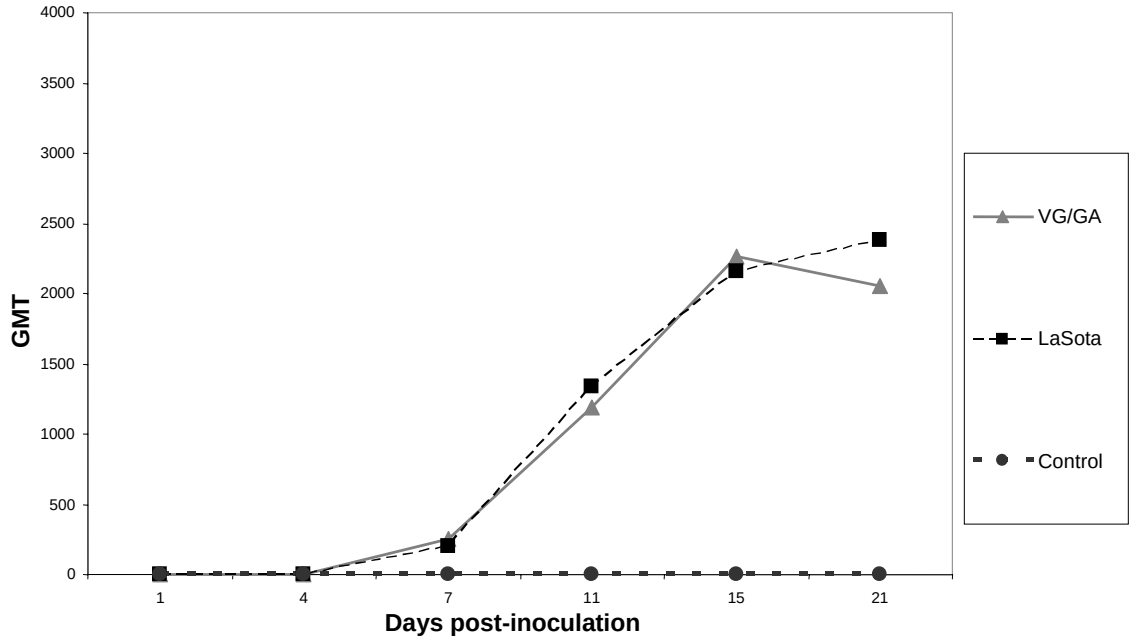
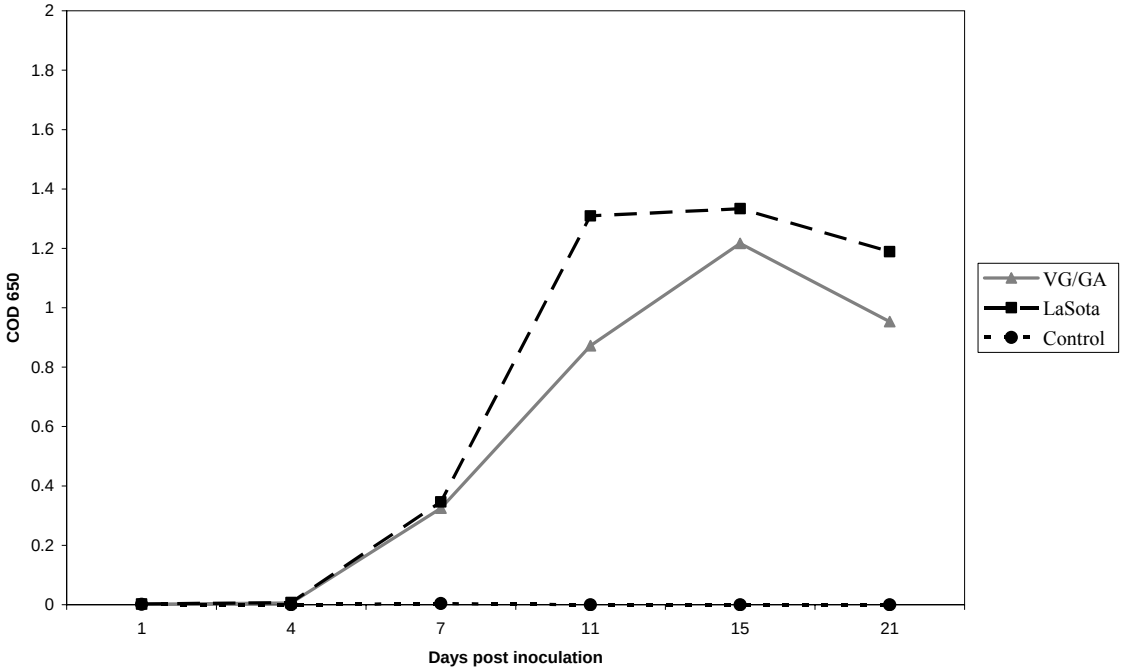
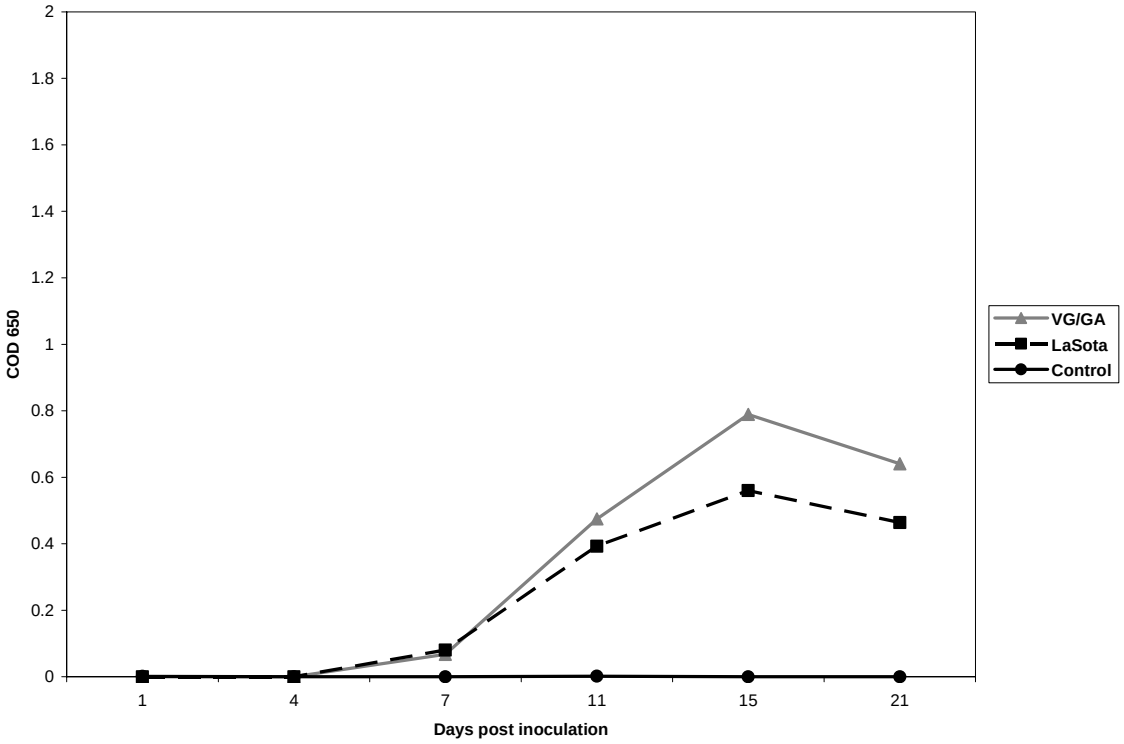


Figure 6.1. Newcastle Disease ELISA serology. Results are expressed as the geometric mean of the ELISA titers. No statistical differences were observed between the vaccine strain. Both differed $P<0.05$ from the nonvaccinated control.

(A)


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
COD 650
1
4
7
11
15
21
Days post inoculation
VG/GA
LaSota
Control

(B)


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
COD 650
1
4
7
11
15
21
Days post inoculation
VG/GA
LaSota
Control

(C)

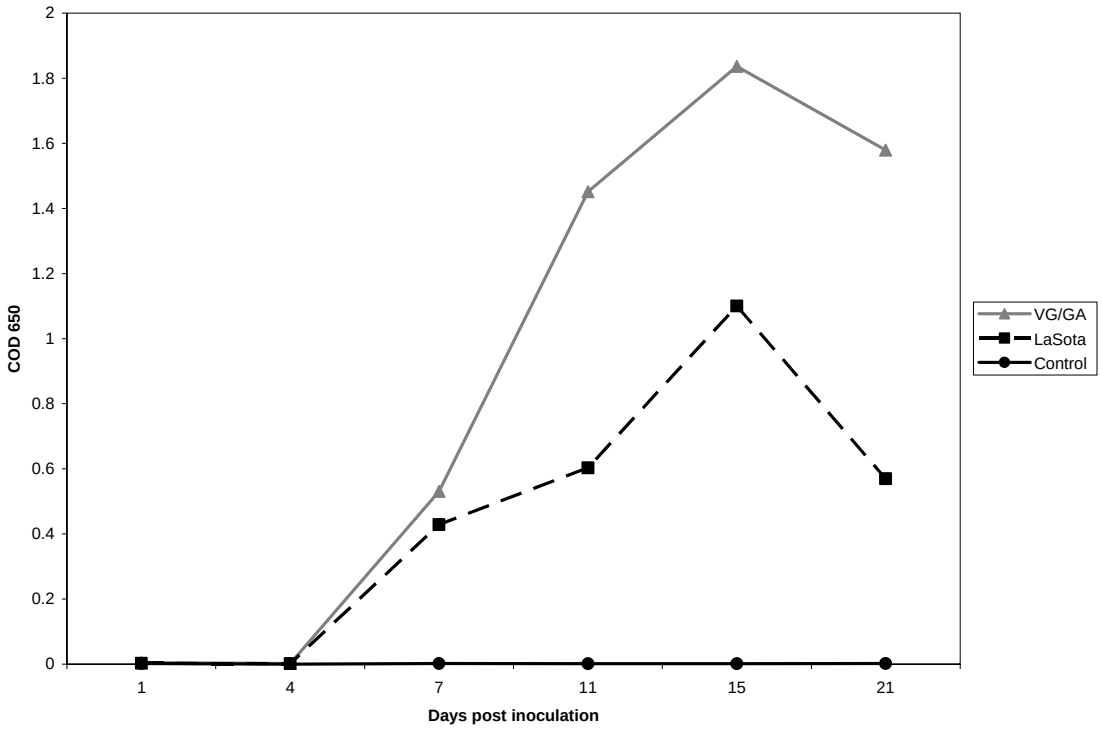


Figure 6.2. Mucosal immunity measured as IgA levels in SPF birds. (A), IgA levels in tracheal washings. (B) IgA levels in intestinal washings. (C) IgA levels in bile. Results are expressed as corrected optical density

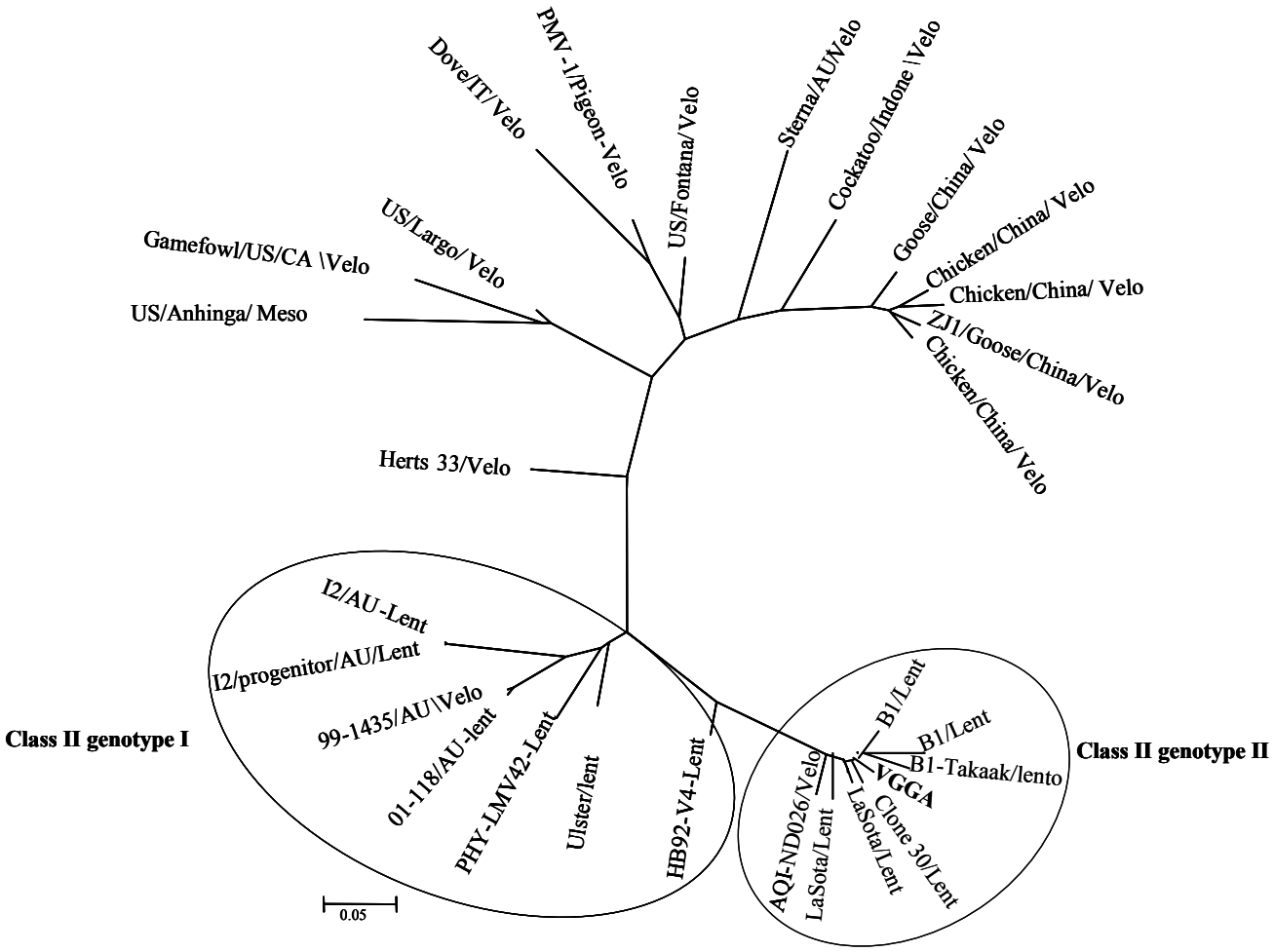


Figure 6.3. Full nucleotide phylogeny of the VG/GA strain of NDV. The VG/GA. strain grouped among the class II genotype II viruses, which correspond with most of the classic poultry vaccine strains.

CHAPTER VII

USE OF FTA® FILTER PAPER FOR THE MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS.

Francisco Perozo, Pedro Villegas, Carlos Estevez, Iván Alvarado & Linda B. Purvis. ***Avian Pathology*. 35(2):93-8. 2006.**
Reprinted here with permission of the publisher.02/022008

Abstract

The feasibility of using FTA® cards to collect allantoic fluid (AF) and chicken tissue samples for Newcastle diseases virus (NDV) molecular detection was evaluated. Trizol ® RNA extraction and one step reverse transcriptase polymerase chain reaction (RT-PCR) were used. FTA® cards allowed NDV identification from AF with titre of $10^{5.8}$ ELD_{50}/ml. The inactivated virus remained stable on the cards for 15 days. NDV was detected from FTA® imprints of trachea, lung, caecal tonsil and cloacal faeces of experimentally infected birds. RT-PCR detection from FTA® cards was confirmed by homologous frozen tissue RT-PCR and virus isolation. Direct nucleotide sequence of the amplified F gene allowed prediction of NDV virulence. No virus isolation was possible from the FTA® inactivated samples, indicating viral inactivation upon contact. The FTA® cards are suitable for collecting and transporting NDV positive samples, providing a reliable source of RNA for molecular characterization and a hazard free sample.

Introduction

Newcastle Disease virus (NDV) is widely distributed and is considered a major concern to the poultry industry (Alexander, 2001; Villegas, 1998a). The virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae,* it is a member of the genus *Avulavirus*, (Mayo, 2002). The molecular basis for NDV pathogenicity has been shown to be highly dependent on the amino acid sequence of the fusion (F) protein cleavage site (Aldous & Alexander, 2001), which has been used as a molecular marker of virulence (OIE, 2004; Panda *et al*., 2004). Nevertheless, strong evidence has arisen that other factors such as the haemagglutinin neuraminidase protein (HN) (de Leeuw *et al*, 2005) and the V protein (Park *et al*., 2003; Zeng *et al*., 2004) may contribute to NDV virulence.

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® card) that is a chemically treated filter paper designed for the collection and room temperature storage of biological samples for subsequent analysis (Natarajan *et al.,* 2000; Rogers & Burgoyne, 2000; Moscoso *et al.,* 2004 ; Smith & Burgoyne, 2004). The FTA® cards have been used for multiple molecular studies such as DNA processing from human or wildlife samples (Raina & Dogra, 2002; Smith & Burgoyne, 2004) and lately it has become a very interesting approach for the detection poultry microorganisms such as Mycoplasma and infectious bronchitis virus *(*Moscoso *et al.,* 2004 *;* Moscoso *et al.,* 2005).

The RT-PCR procedure has been established as a reliable tool for NDV detection in allantoic fluid and in poultry vaccines (Stäuber *et al*., 1995; Farsang *et al*., 2003). The detection

of NDV in fresh feces and tissues by RT-PCR has also been described (Gohm *et al*., 2000; Aldous & Alexander, 2001). Molecular detection and characterization of NDV is not commonly performed on chemically inactivated samples due to reports of RNA modifications and problems in nucleic acid extraction, which compromise the yield of high quality DNA or RNA (Coombs *et al*., 1999; Masuda *et al*., 1999). A virus inactivation process able to ensure high quality RNA for molecular pathotyping, would be an improvement in field sampling and shipping of NDV for diagnosis means. In this study, the feasibility of using FTA® cards for sampling, inactivation and virus detection from allantoic fluid (AF) and tissue samples by RT-PCR was assessed.

Materials and Methods.

Virus. LaSota NDV strain vaccine (Merial Select, Inc. Gainesville, Georgia. USA) was propagated by inoculation into embryonating chicken eggs, as previously described (Senne, 1998). The allantoic fluid (AF) was collected and tested using rapid plate hemagglutination of 5% chicken red blood cells (Alexander, 1998). Virus titration was performed as previously described (Villegas, 1998b). The titre obtained was $10^{8.8}$ EID_{50}/ml. The AF stock virus was stored at – 80 °C until needed.

RNA extraction and amplification. Following manufacturer recommendations, two RNA extraction procedures and two amplification protocols were used to determine the best extraction/amplification method for FTA® detection of NDV. A: High pure RNA isolation kit (Roche Diagnostics Co., Indianapolis, IN) + one step RT-PCR (Titan Kit- Roche Diagnostics Co). B: Trizol ® (Life Technologies Inc., Grand Island, USA) + one step RT-PCR. C: High pure RNA isolation kit + two step RT-PCR (SuperScript III/Failsafe®). D: Trizol ® + two step RT-

PCR. Degenerate primers designed to amplify a region of the F gene that includes its cleavage site were used (NDV-F328: 5'-TGGTGAITCTATCCGIAGG-3', NDV-R581: 5'-CTGCCACTGCTAGTTGIGATATACC-3') (Seal *et al.,* 1995). RT-PCR tests were carried out in a My Cycler thermocycler (BIO-RAD, Hercules, USA) with incubation for 45 minutes at 48 °C for RT, heating at 94 °C for 2 minutes and 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds and polymerization at 68 °C for 60 seconds with a final elongation step of 7 minutes at 68 °C. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide with a concentration of 0.5 µg/ml.

Sensitivity and stability of FTA® / RT-PCR system. Serial ten fold dilutions up to 10^{-9} were made from the initial AF stock ($10^{8.8}$ EID_{50}/ml) to evaluate the detection sensitivity of FTA® cards for NDV from positive AF. For each one of the nine dilutions and the undiluted AF, 50 µl were applied to the four matrix circles present in the FTA® cards. After 24 hours, 25 punches were taken from one matrix circle of each card, using a 2-millimeter puncher (Harris Micro-Punch™ Fisher Scientific, Pittsburgh. USA) in order to recover the surface covered by the 50 µl added. RT-PCR reactions were run for each sample to determine the highest dilution where viral RNA was detectable. RT-PCR reactions (50 µl) of the same AF dilutions applied to the cards were run parallel as a control. Further evaluation of the sensitivity was performed by decreasing the number of punches, using 25, 20, 15, 10, 5 and 1. Virus identification by RT-PCR was attempted on days 1, 7, 14 and 30 after sample collection from cards stored at room temperature (approx. 25 °C) to evaluate the stability of viral RNA on the FTA® cards by looking for amplification efficiency over time, as judged visually on ethidium bromide stained gels.

Organ selection for FTA® / RT-PCR detection of NDV and virus isolation. Ten 7-day old specific pathogen free chicks (Merial Select, Inc. Gainesville, Georgia, USA) were used to select the best-suited tissues for RT-PCR detection of NDV from FTA® cards. Seven birds were inoculated conjunctival-orally with 100 µl of the stock virus ($10^{8.8}$ ELD_{50}/ml); the remaining three birds were used as control and were inoculated with 100 µl of phosphate buffered saline (PBS). Starting on day one post- inoculation (p.i.), one chicken was euthanized every day for 7 days. One control bird was euthanized on days 1, 4 and 7. From each bird, faecal samples and tracheal swabs were smeared on the cards using sterile cotton tipped applicators. Tissue samples (approx. 1 cm^2) from heart, kidney, trachea, spleen, proventrículus, brain, lung and caecal tonsils were collected from the birds following previously recommended protocols (Gohm *et al*., 2000). An imprint was made by gently pressing the tissue against the provided matrix area of FTA® cards, as described by Higgins *et al*. (2000). The remaining tissues where stored at -80 °C until processing. After 24 hours, RT-PCR was performed on both card and frozen tissue samples. To confirm the RT-PCR results obtained from FTA® cards, virus isolation was attempted from samples obtained on day 2 p.i. (Alexander, 1998).

***In vivo* experiment.** Specific pathogen free chickens (44) were hatched and reared in isolation. At 3 weeks of age, 4 chickens were bled for antibody titre determination by enzyme-linked immunosorbent assay (ELISA), using FlockCheck® Newcastle disease antibody test (IDEXX , Maine, USA) and euthanized for necropsy and tissue collection as described later. The remaining 40 birds were separated in two groups of 20 chickens each. One group was inoculated conjunctival-orally with 100 µl of the stock NDV virus ($10^{8.8}$ ELD_{50}/ml). After 2 days, the non-inoculated group was wing banded and added to the inoculated group as contact birds. Two inoculated birds were euthanized daily through day 6, thereafter on days 8, 10 and 12 p.i. Two

contact birds were euthanized on the same days as the inoculated animals, starting on day 4 until day 12. From each bird, trachea, lung, caecal tonsil, and cloacal faecal samples were collected and applied to the cards for RT-PCR analysis. Prior to euthanasia, experimentally infected birds were bled to measure their antibody levels p.i. or exposure to the virus using ELISA.

Sequencing of the F gene. RT-PCR amplified fragments containing the 250 base pairs (bp) portion of the F gene were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) using manufacturers' recommendations. Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as described by the manufacturer. Sequencing reactions were run in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed with the aid of the DNAStar software (DNAStar, Inc., Madison, WI).

Virus inactivation by FTA® cards. AF and trachea samples positive for NDV FTA® imprints were allowed to elute in FTA® purification reagent (Whatman International, Ltd. UK). One hundred microliters of this solution were inoculated in the chorioallantoic sac of 9-day specific pathogen free embryos. After 5 days, the AF was collected and tested using the rapid plate hemagglutination test with 5% chicken red blood cells (Alexander, 1998). Virus detection by RT-PCR was also attempted from the collected AF.

Results.

RNA extraction and amplification. The overall average RNA yield obtained from the different dilutions using Trizol ® extraction was 58.9 µg/ml and for the High pure RNA kit procedure was 20.1 µg/ml. The RT-PCR amplification after Trizol® treatments was more sensitive (a tenfold difference in detection) than the treatments using the High pure RNA kit. The use of FTA® cards

for Newcastle disease virus detection from allantoic fluid and the comparison between different RNA extraction and amplification methods are showed in Table 1.

Sensitivity and stability of FTA® / RT-PCR system. The lowest concentration at which nucleic acid amplification from the FTA® cards occurred was $10^{5.8}$ ELD_{50}/ml. The detection level of NDV nucleic acids was always higher when AF prior to its inactivation was used as study material in comparison with the level of detection observed for the FTA® cards (1 log_{10} difference). The effect of decreasing the amount of RNA template on RT-PCR detection of NDV (by decreasing the number of punches from the FTA® cards) was observed as a weakening of the amplification signal, judged visually on ethidium bromide stained gels. RT-PCR allowed the detection of NDV nucleic acids from the cards even when only one punch was used. The stability of viral RNA on FTA® cards was measured by performing RT-PCR on ten fold serial dilutions of the card inactivated AF at days 1, 7, 14 and 30 after collection. The detection sensitivity decreased one log_{10} by day 14.

Organ selection for FTA® / RT-PCR detection of NDV and virus isolation. RT-PCR positive results were obtained in the organ selection experiment from trachea, lung, caecal tonsils and cloacal faeces (Figure 1). The trachea was positive in all infected birds up to day 6 p.i., caecal tonsils tested positive on days 6 and 7 pi, and cloacal faeces samples were positive only on days 1 and 2 p.i. No amplification was obtained at any time point from control birds. Samples of heart, kidney, spleen, proventriculus, brain, or tracheal swabs from experimentally infected birds, rendered negative results to RT-PCR analysis at all time points. Meanwhile, RT-PCR procedures performed on homologous frozen tissues were positive for trachea, lung, caecal tonsils and cloacal faeces at the same time points as the card inactivated samples. Negative tissues were negative for both the frozen tissues and FTA® card inactivated samples. Virus isolation attempted

on the frozen samples from day 2 p.i. was positive for trachea, lung and faeces, confirming the presence of the virus detected by the RT-PCR procedure.

***In vivo* experiment.** The RT-PCR results for the inoculated and contact birds are shown in Table 2. Trachea and faeces showed the first positive RT-PCR results (day 1 p.i.). The trachea remained positive longer (6 days p.i.). In the contact animals, the first positive signals were found in the trachea on day 3 after exposure, molecular detection was not possible from FTA® inactivated tracheal swabs. No single organ was consistently positive for all the days tested. No clinical signs were observed in the inoculated or contact birds. Pre-inoculation serum samples were all NDV antibody negative, seroconversion of the inoculated animals was observed 6 days p.i. The contact birds seroconverted 7 days after exposure. The highest titres were recorded on day 12 p.i. with an ELISA geometric mean titre of 11,995 for the inoculated birds and 1,553 for the contact group.

Sequencing of the F gene. After analysis of the sequenced data, no differences were observed in the alignment of the amplified nucleotides obtained from the FTA® cards when compared with the un-inactivated AF samples RT-PCR products (data not shown). Sequencing of the 250 bp portion of the NDV F gene, allowed the prediction of the amino acid sequence at the F0 cleavage site, which as expected was found to correspond to a lentogenic virus.

Inactivation of NDV on FTA® cards. The AF obtained from embryos inoculated with a FTA® card eluate, failed to hemagglutinate chicken red blood cells and was negative to NDV/ RT-PCR detection. No amplicons were observed from the FTA® card inactivated fluid after RT-PCR analysis. These results indicate complete inactivation of the NDV on contact with the FTA® cards.

Discussion.

Tests using live virus, such as mean death time, intracerebral and intravenous pathogenicity index are biological tests required to determine the isolates' pathotypes to confirm an outbreak of Newcastle disease (Alexander, 1998; OIE, 2004). Meanwhile, the molecular approach for NDV identification and pathotyping using direct sequencing of F protein gene cleavage site is accepted as a pathotyping procedure and has been used for surveillance of NDV (Seal, et al., 1995; Marin *et al.,* 1996; OIE, 2004; Panda *et al*., 2004; Zeng *et al*., 2004). RT-PCR and direct nucleotide sequencing are not available in some countries or regions, so samples need be transported in a safe way to laboratories with those capabilities, following high standards of biosecurity during shipping (Snyder, 2002). We report the feasibility of using FTA® inactivated NDV isolates for diagnosis and molecular pathotyping avoiding the risks of handling and processing live viruses.

A comparison between 4 different extraction/amplification procedures was made to select the most suitable protocol for NDV identification on FTA® cards. No difference on detection sensitivity was observed when one or two steps RT-PCR were used. The one step RT-PCR is a simpler method, so it was selected to be used in combination with the Trizol ® extraction protocol, which yielded higher RNA levels than the High pure RNA isolation kit. This agrees with previous reports recommending the use of Trizol ® over other RNA extraction procedures (Wex *et al.,* 2003).

The FTA® cards for NDV sampling and inactivation coupled with RT-PCR, allowed the detection of virus from AF with a titre of $10^{5.8}$ ELD_{50}/ml, (a 10^{-3} dilution of the $10^{8.8}$ ELD_{50}/ml viral stock). Higher sensitivity of the RT-PCR test for AF and NDV live vaccines (detection of titres as low as 5×10^2 ELD_{50}) has been previously reported (Stäuber *et al*., 1995; Gohm *et al*., 2000). These results differ from the $10^{4.8}$ ELD_{50}/ml reported here for the control samples (direct

RNA extraction from AF). A lower amount (50 µl) of initial template used in this trial to match the AF sampled on the cards when compared with previous studies, may explain the RT-PCR decrease in sensitivity. Further differences in sensitivity observed in the RT-PCR for the FTA® inactivated fluids when compared with the control samples, may be due to a detrimental effect of FTA® inactivation on the viral RNA, as previously reported for other chemical inactivated samples (Coombs, 1999; Masuda *et al*., 1999).

The RT-PCR identification of NDV in stored FTA® cards was possible after 30 days, although a decrease in sensitivity was observed after 14 days of storage at room temperature. The decrease in sensitivity over time has been previously explained as a consequence of RNA denaturation by formation of nicks on the RNA strands (Rogers & Burgoyne, 2000; Doobs *et al*., 2002; Moscoso *et al*., 2005).

The nucleic acids of the NDV inactivated on FTA® cards from experimentally and contact-infected chickens were detected by RT-PCR, as early as 1 day p.i. in experimentally infected chickens, but serological detection was not possible until day 6 p.i. These results emphasize the importance of molecular detection as diagnostic tool in NDV surveillance (Aldous & Alexander, 2001). The trachea was the most suitable organ for NDV detection from the FTA® cards imprints, but no amplification from FTA® inactivated tracheal swabs was possible, although tracheal and cloacal swabs are commonly recommended for virus isolation during field outbreaks (Alexander, 1998; Alexander, 2001). This represents the drawback that for NDV detection from FTA® cards, the birds would need to be euthanized in order to collect the sample. The failure to identify the virus in the trachea swabs may be related to the amount of viral RNA present in the swab, when compared with the amount of virus obtained from a tissue imprint, where epithelial cells actively targeted by virus replication remain over the FTA® cards matrix.

The organ selection for FTA® sampling might be affected by NDV strain tropism, age and physical conditions of the birds and even immunological aspects such as maternal antibodies or the presence of immunosuppressive factors (Alexander, 1998; Villegas 1998a; Alexander, 2001). In this trial, NDV specific pathogen free chickens were experimentally infected, therefore, no interference of maternal antibodies with the virus pathogenesis was expected and the LaSota strain used has a high rate of replication in respiratory tract which may explain the prevalence of the virus in trachea (Alexander, 1998).

Amplification of a specific cDNA segment has been reported from inactivated oil-adjuvanted NDV vaccines without prior treatment (Stäuber *et al*., 1995). Nevertheless, inactivated samples are not commonly used in NDV molecular detection and pathogenicity studies due to the difficulties generated by the chemical inactivation procedures, which can impair the quality of the nucleic acids, jeopardizing the reliability of the tests (Coombs *et al*., 1999; Masuda e*t al.*, 1999). The sensitivity of the RT-PCR detection from the FTA® cards inactivated tissues proved to be the same as that obtained by RT-PCR from frozen tissues and for virus isolation. Therefore, we have demonstrated the feasibility of using FTA® cards for direct sampling and inactivation of tissues and faeces from NDV infected animals for RT-PCR detection.

Nucleotide sequencing of the amplified 250 bp segment of the F gene allowed molecular pathotyping of the inactivated NDV, representing a useful tool for the surveillance of the disease in areas where molecular or biological pathotyping of the field isolates is not possible.These results are in agreement with previous reports on molecular pathotyping of NDV (Seal *et al*., 1995; Marin *et al.,* 1996; Panda *et al*., 2004).

The RT-PCR procedures failed to identify viral RNA from the embryos inoculated with a FTA® elute, which means no virus re-isolation from the cards was possible. This result agrees with previous publications, where the complete inactivation of the microorganisms by the FTA® card was reported (Raina & Dogra, 2002; Smith *et al*., 2004; Moscoso *et al*., 2005). The FTA® card sampling and inactivation procedure used in this trial can provide an improvement in NDV analysis protocols. It allows virus identification and molecular pathotyping direct from the bird, avoiding time consuming virus isolation and amplification steps, simplifying the field work and diminishing the risk of handling live viruses.

Acknowledgements.

The authors will like to thank to Dr. Mark Jackwood and Dr. Stanley Kleven, University of Georgia, Poultry Diagnostic and Research Center, for reviewing this manuscript.

REFERENCES

Aldous, E. & Alexander, D. (2001). Detection and differentiation of Newcastle disease virus (avian paramixovirus type 1). *Avian Pathology, 30*, 117-128.

Alexander, D. (2001). Gordon Memorial Lecture. Newcastle disease. *British. Poultry. Science*, *42*, 5-22.

Alexander D.J. (1998). Newcastle Disease Virus and other Avian Paramyxoviruses. In: D. E. Swayne, (Ed.). *A Laboratory Manual for Isolation and Identification of Avian Pathogens*, 4th ed. (pp. 235-240). Kennett Square, PA: The American Association of Avian Pathologists.

Coombs, N., Gough, A., & Primrose, J. (1999). Optimization of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Research, 27*, 2223-2226.

de Leeuw, O. Koch, G., Hartong, L., Ravenshort, N. & Peeters, B. (2005). Virulence of Newcastle disease virus is determined by the cleavege site of the fusion protein and by both the stem region and globular head of the haemagglutinin-nuraminidase protein. *Journal of General Virology*, 86, 1759-1769.

Dobbs, L., Madigan, M., Carter, A. & Earls L. (2002). Use of FTA gene guard filter paper for the storage and transportation of tumor cells for molecular testing. *Archives of Pathology and Laboratory Medicine, 126*, 56-63.

Farsang, A., Wehmann, E., Soós, T. & Lomniczi, B. (2003). Positive identification of Newcastle disease virus vaccine strains and detection of contamination in vaccine batches by restriction site analysis of the matrix protein gene. *Journal of Veterinary Medicine*, *50*, 311–315.

Gohm, D., Thür, B. & Hofmann, M. (2000). Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. *Avian Pathology, 29*,143-152.

Higgins, J., Hubalek, Z., Halouzka, J., Elkins, K., Sjostedt, A., Shipley, M. & Ibrahim, M. (2000). Detection of *Francisella tularensis* in infected mammals and vectors using a probe based polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, *62*, 310-318.

Marin, M., Villegas, P., Bennett, J.D. & Seal, B.S. (1996).Virus characterization and sequence of the fusion protein gene cleavage site of recent Newcastle disease virus field isolates from the southeastern United States and Puerto Rico. *Avian Diseases*, *40*, 382-90.

Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M. & Okubo K. (1999). Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Research*, *27*, 4436-4443.

Mayo, M. (2002). Virus taxonomy-Houston 2002. *Archives of Virology, 147*, 1071-1076.

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

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

Natarajan, P., Trinh, T., Mertz, L., Goldsboroug, M. & Fox, D. (2000). Paper-based archiving of mammalian and plant samples for RNA analysis. *Biotechniques*, *29*, 1328-33.

OIE (2004). Newcastle Disease. *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (pp.161-169). Paris: Office International des Epizooties.

Panda, A., Huang, Z., Elankumaran, S., Rockeman, D. & Samal, S. (2004). Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microbiology Pathology*, *36*, 1-10.

Park, M., Shaw, M., Munoz-Jordan, J., Cros, J., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. & Blaser, C. (2003). Newcastle disease virus (NDV)-based assay demonstrates interferon antagonist activity for the NDV V protein and Nipah virus V, W, and C proteins. *Journal of Virology, 77*, 1501-1511.

Raina, A. &. Dogra, T. D. (2002). Application of DNA fingerprinting in medicolegal practice. *Journal of Indian Medical Association*, *100*, 688-94.

Rogers, C. & Burgoyne L. (2000). Reverse transcription of an RNA genome from databasing paper (FTA®). *Biotechnology and Applied Biochemistry*, *31*, 219-24.

Seal, B. S., King, D. J. &. Bennett J.D. (1995). Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotypes prediction and molecular epidemiological analysis. *Journal of Clinical Microbiology*, *33*, 2624-30.

Senne, D. A. (1998). Virus propagation in embryonated eggs. In: D. E. Swayne, (Ed.). *A laboratory Manual for Isolation and Identification of Avian Pathogens*, 4th ed. (pp. 235-240). Kennett Square, PA: The American Association of Avian Pathologists.

Smith, L. M. & Burgoyne L. A. (2004). Collecting, archiving and processing DNA from wildlife samples using FTA data basing paper. *BMC Ecology*, *4*, 4-9.

Snyder, J. (2002). Packaging and shipping of infectious substances (Newsletter). *Clinical Microbiology Newsletter,* Elsevier Science Inc., NY. 12, 89-92.

Staüber, N., Brechtbuhl, K., Bruckner, L. & Hofmann, M. (1995). Detection of Newcastle disease virus in poultry vaccines using the polymerase chain reaction and direct sequencing of amplified cDNA. *Vaccine, 13*, 360-364.

Villegas, P. (1998a). Viral diseases of the respiratory system. Poultry Science, *77*, 1143-1145.

Villegas, P. (1998b). Titration of biological suspensions. In: D. E. Swayne, (Ed.). *A laboratory Manual for Isolation and Identification of Avian Pathogens*, 4th ed. (pp. 248-253). Kennett Square, PA: The American Association of Avian Pathologists.

Wex, T., Treiber.G., Lendeckel. U. & Malfertheiner. P. (2003). A two step method for the extraction of high-quality RNA from endoscopic biopsies. *Clinical Chemistry and Laboratory Medicine. 41*, 1033-1037.

Zeng, J., Fournier, P. & Schirrmacher V. (2004). High cell surface expression of Newcastle disease virus proteins via replicon vectors demonstrates syncytia forming activity of F and fusion promotion activity of HN molecules. *International Journal of Oncology. 25*, 293-302.

Table 7.1. *Use of FTA® cards for Newcastle disease virus detection from allantoic fluid and comparison between different RNA extraction and amplification methods.*

RT-PCR amplifications (Initial AF concentration $10^{8.8}$ ELD_{50}/ml)								
	FTA® card				AF			
Virus dilutions	A	B	C	D	A	B	C	D
10^{0}	+	+	+	+	+	+	+	+
10^{-1}	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+
10^{-3}	-	+	-	+	+	+	+	+
10^{-4}	-	-	-	-	-	+	-	+
10^{-5}	-	-	-	-	-	-	-	-
10^{-6}	-	-	-	-	-	-	-	-
10^{-7}	-	-	-	-	-	-	-	-
10^{-8}	-	-	-	-	-	-	-	-
10^{-9}	-	-	-	-	-	-	-	-

A= High Pure RNA kit + one step RT-PCR. C= High Pure RNA kit + two steps RT-PCR .

B= Trizol ® + one step RT-PCR. D= Trizol ® + two steps RT-PCR.

(+) = NDV (RT-PCR) 250 bp amplicon. (-) = No amplification.

Table 7.2. *Detection of NDV in selected FTA® cards samples after in vivo infection with LaSota strain of NDV.*

	Organ	Animal group	Days P.I.								
			1	2	3	4	5	6	8	10	12
Inoculated	Trachea	A	+[a]	+	+	+	+	+	-	-	-
		B	+	+	+	-[b]	+	-	-	-	-
	Lung	A	+	-	-	-	+	-	-	-	-
		B	-	-	+	-	-	-	-	-	-
	Caecal	A	-	-	-	-	-	+	+	-	-
	Tonsil	B	-	-	-	-	-	+	-	-	-
	Cloacal	A	+	+	-	-	-	-	-	-	-
	Feces	B	+	+	-	-	-	-	-	-	-
Contact infected	Trachea	A	-	-	-	-	+	+	-	-	-
		B	-	-	-	-	-	+	+	-	-
	Lung	A	-	-	-	-	-	-	-	-	-
		B	-	-	-	-	-	-	-	-	-
	Caecal	A	-	-	-	-	-	-	-	+	-
	Tonsil	B	-	-	-	-	-	-	-	+	-
	Cloacal	A	-	-	-	-	+	-	-	-	-
	Feces	B	-	-	-	-	+	+	-	-	-

Samples from two inoculated and two contact birds were taken each day (A and B respectively). Contact birds were added at day 2 p.i. and sampled started at day 4 p.i. ending day 12. [a] RT-PCR positive signal (250 bp amplicon). [b] RT-PCR negative signal (no amplicon).

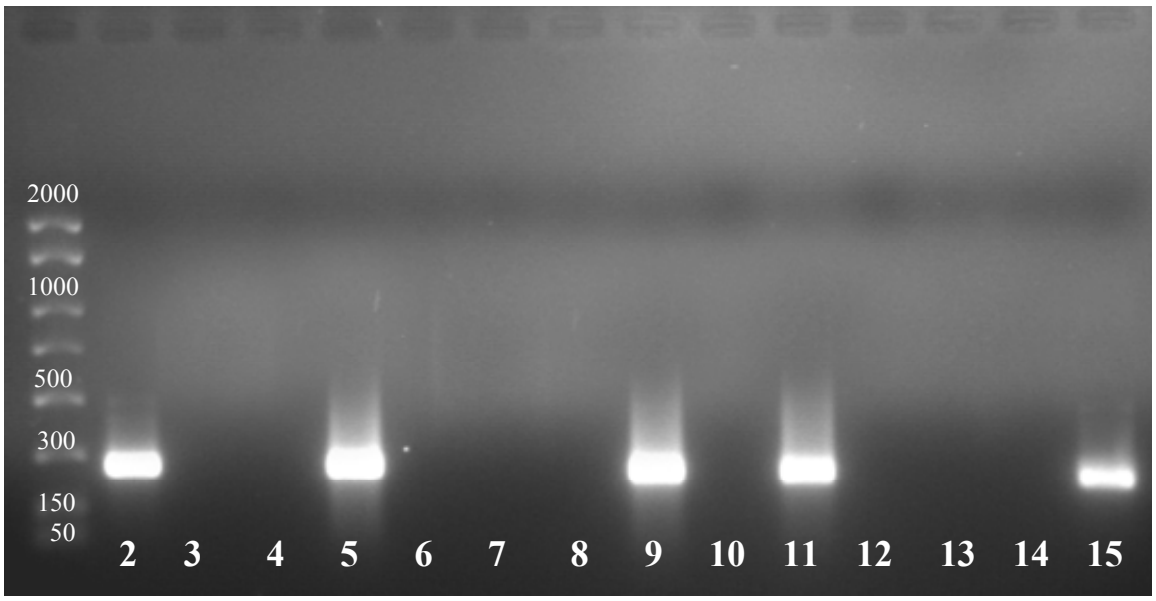


Figure. 7.1. *Gel electrophoresis of RT-PCR of FTA® cards tissue imprints from experimentally and control birds. Lane 1 = DNA PCR marker (Amresco® Ohio, USA). Lane 2= cloacal faeces. Lane 3= heart. Lane 4= kidney. Lane 5= trachea. Lane 6=spleen. Lane 7= proventriculus. Lane 8= brain. Lane 9= lung. Lane 10= tracheal swab. Lane 11= caecal tonsil. Lane12= control bird trachea. Lane13= control bird lung. Lane14= control bird caecal tonsil. Lane 15=positive control (AF).*

CHAPTER VIII

CONCLUSIONS

Conclusions on the Avian adeno-associated virus based protein expression for poultry vaccination:

- The generation of rAAAV particles expressing the Newcastle disease virus HN protein was demonstrated by immunocytochemistry, electron microscopy and western blot analysis. Serological evidence of *in vivo* expression of the recombinant protein was obtained in SPF birds (ELISA, HI, IF, VN).

- Protection against virulent challenge was observed in broilers vaccinated with the rAAAV particles expressing the HN protein. Hence, the feasibility of developing and using an avian adeno-associated virus based gene delivery system that represents a new vaccine candidate for NDV was demonstrated.

- The feasibility of generating recombinant avian adeno-associated virions expressing the immunogenic viral protein 2 (VP2) of IBDV was demonstrated. A detectable systemic immune response measured as antibodies against IBDV was elicited in SPF birds after inoculation with the recombinant virus.

- The rAAAV based transgenic expression of the IBDV VP2 protein in SPF birds induces protective immunity against IBDV virulent challenge and vaccination with the rAAAV-VP2 virions do not affect bursal lymphoid tissue, demonstrating its potential for use in young chickens.

Conclusions on the characterization of the VG/GA strain of Newcastle disease virus:

- The VG/GA strain of NDV can be detected RT-PCR at different time points after infection both in the respiratory and intestinal tract of chickens.

- The replication pattern of VG/GA strain induced a stronger localized mucosal immune response in the intestinal tract shown by an increased production of NDV-specific IgA. This feature may represent a competitive advantage in the event of a velogenic viscerotropic challenge where the virus have been reported to induce massive destruction of intestinal lymphoid areas and extensive ulceration of overlying intestinal epithelium associated with active viral replication.

- No significant differences were observed in the levels of total (unspecific) IgA between the nonvaccinated control and the vaccinated groups, suggesting that there is no measurable effect of NDV vaccination in the overall IgA load of the mucosal tissue in chickens.

- Despite the presence of the maternal antibodies in broilers, early vaccination with the VG/GA strain afforded 95 to 100% protection against lethal challenge, equivalent to the protection offered by LaSota strain. These results validate anecdotic data obtained from field observations and confirm results from vaccine-challenge trials performed in SPF chickens and quails.

- The efficacy of the different treatment combinations using both VG/GA and LaSota strains demonstrated the feasibility of using a multiple strain vaccine protocol with VG/GA strain for initial vaccination when high challenge is present and field revaccination is scheduled.

- Based on how fast the VG/GA is cleared from the respiratory tract after priming the mucosal immune response, the mucosal IgA production both in the respiratory and the intestinal tract and the levels of protection afforded by single or multiple doses of the vaccine, initial vaccination with the VG/GA strain may be advantageous for the integrity of the respiratory mucosa of young chickens when multiple vaccination and/or field exposure is expected.

- After full genome sequence analysis, the VG/GA strain was grouped within the class II, genotype II of Newcastle disease viruses.The VG/GA phylogeny differed from the other vaccine strains able to replicate in the intestine; these strains belong to the class II but in the genotype I. The Ulster, QV4 derivates, and PHY-LMV42 strains are more closely

related than the VG/GA to the Australian isolates for which drift mutations at the cleavage site responsible for increases in virulence has been reported.

- The changes observed in proteins associated with tissue tropism may explain the differential phenotype of the VG/GA strain; however, further studies including the generation of a reverse genetic system and nucleotide substitution studies are required to verify the significance of these changes.

Conclusions on the evaluation of FTA® cards for NDV sampling and detection:

- FTA® cards allowed NDV identification from AF with titre of $10^{5.8}$ ELD_{50}/ml. The inactivated virus remained stable on the cards for 15 days.

- NDV was detected from FTA® imprints of trachea, lung, caecal tonsil and cloacal faeces of experimentally infected birds, the RT-PCR detection from FTA® cards was confirmed by homologous frozen tissue RT-PCR and virus isolation.

- Direct nucleotide sequence of the amplified F gene allowed prediction of NDV virulence. No virus isolation was possible from the FTA® inactivated samples, indicating viral inactivation upon contact.

- The FTA® cards are suitable for collecting and transporting NDV positive samples, providing a reliable source of RNA for molecular characterization and a hazard free sample.